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LARS LINDQVIST

A flash photolysis study of fluorescein

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A FLASH PHOTOLYSIS STUDY OF FLUORESCEIN

INAUGURAL DISSERTATION

by

LARS LINDQVIST

FIL. LIC., ÖSTG.

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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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With 48 figures in the text

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Introduction

The purpose of the present investigation was to provide information about the reaction mechanisms involved in photosensitized reactions, particularly in relation to photoreduction and photo-oxidation processes. The xanthene dyes constitute an important group of sensitizers, capable of inducing reduction as well as oxidation, and a representative of this group, fluorescein, was chosen for this study. Fluorescein

has not been as extensively used as sensitizer as some of its derivatives, taking eosin as an example, but was preferred in the present case because information about the triplet state of this dye is available.

The photoreactions of xanthene dyes, until very recently, have been studied only by indirect methods and hypothetical reaction mechanisms have been put forward to explain the experimental results. However, with the development of the flash photolysis method [1], the direct study of photochemical reactions has become possible, and this method has been used exclusively in the present investigation. Since this work was initiated, two short communications about the flash photolysis of eosin have appeared [2, 3].

The use of the flash photolysis method in the study of photoactive systems is limited by the flash light energy, determining the degree of conversion to activated species, as well as by the flash duration, determining the time resolution of the kinetics. The development of a very intense flash light source of short flash duration, by Claesson and the author [4], provided the basis for the present investigation. The reactions following flash excitation were studied by kinetic spectrophotometry. The flash photolysis apparatus is described in Chapter 1 and other experimental details in Chapter 2. A detailed knowledge of the forms in which fluorescein appears in water solution was required for the photochemical study, Therefore, in a complementary investigation the electrolytic dissociation and the dimerization of fluorescein were studied, reported in Chapter 3. In Chapter 4 the experimental results of the kinetic investigation are presented, followed by a general discussion in Chapter 5.

Chapter 1. Flash photolysis apparatus

Flash light source

The flash light source used has been described in detail in [4], and only some pertinent data will be given in this connection.

The light source consists of two straight quartz discharge tubes, connected in series to a condenser bank. The lamps, constructed at the Institute, are provided with tungsten electrodes which are separated by a distance of 60 cm. The lamps are filled with oxygen to a pressure of about 5 mm Hg, permitting them to be subjected to the full condenser voltage without firing spontaneously. The discharge is initiated by the application of a high voltage pulse on the two interconnected electrodes. The two lamps are placed symmetrically around the sample to be illuminated, as shown in Fig. 3. A magnesium oxide coated reflector surrounds the lamp pair.

The condenser bank has a capacity of 1370 μF, storing an electrical energy of 33,600 J at the maximum voltage, 7 kV. The double voltage is applied across the series-connected lamps, by arranging the condensers into two halves, charging one half positively and the other negatively.

At the highest energy dissipated, the flash duration time ($1/e$ time) is about 150 μsec. In most of the experiments a capacity of 400 μF, at 5.5 kV, was used. The corresponding light intensity-versus-time curve at 490 mμ is shown in Fig. 1.

Only the visible part of the emission spectrum of the photolysis flash was utilized for excitation. This spectral region was isolated by means of a liquid filter, consisting of a mixture of ferric alum and copper sulphate, 10 g $(NH_4)_2SO_4 \cdot Fe_2(SO_4)_3 \cdot 24\,H_2O$,

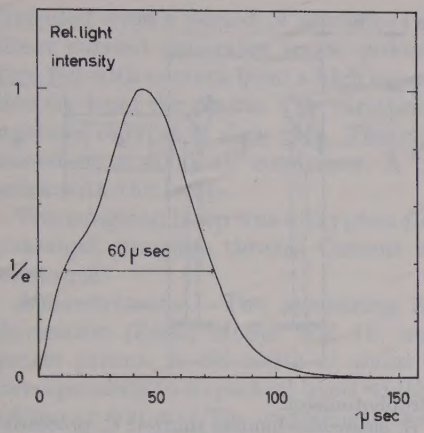


Fig. 1.

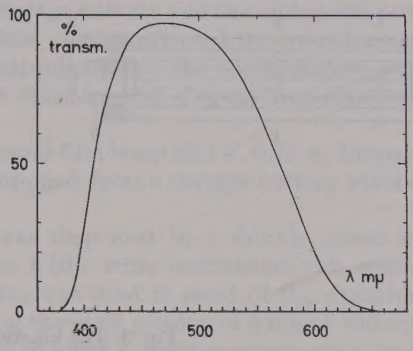


Fig. 2.

Fig. 1. The light intensity of a 6000 J flash (400 μF, charged to 5.5 kV) as a function of time, measured at 490 mμ. Flash duration time ($1/e$ time) 60 μsec. Time resolution of the measurement 1 μsec.

Fig. 2. Light transmission of the ferric alum-copper sulphate filter utilized for isolating the visible part of the flash light spectrum. Optical path 1 cm.

200 g $CuSO_4 \cdot 5H_2O$ and 5 ml H_2SO_4 (conc.) per liter. A layer of the filter liquid, 1 cm thick, surrounded the solution to be illuminated. Fig. 2 shows the transmittance of the filter at an optical path of 1 cm.

The amount of light incident on the cuvette could be varied by changing the capacity or the voltage of the flash lamp condensers. When it was preferable not to change the flash intensity contour, gray filters were used as an alternative. A set of flat, fine-mesh screens, which were positioned between the flash lamps and the cuvette, were available. The transmittance of the filters was about 45, 20 and 10 %, as determined by uranyl oxalate actinometry.

No measurements of the light emitted in the wavelength region of fluorescein absorption were made. Determinations of the light absorbed by uranyl oxalate and potassium ferrioxalate actinometers are, however, reported in [4]. The difference between the values obtained with the two actinometers gives the light energy in the region 350 to 480 mμ. For an electrical energy of 6000 J (400 μF, 5.5 kV) an average flux incident on the cuvette amounting to 0.02 einstein cm^{-2} sec^{-1} is calculated.

Kinetic spectrophotometer

The kinetics of the reactions occurring in the flash illuminated solutions were studied exclusively by measuring the light absorption changes of the reacting system. For this purpose a single-beam absorption spectrophotometer with quartz optics was constructed, the essential parts of which are outlined in Fig. 3.

Particularly long reaction cells were used (60 cm optical path), allowing measurements on very weakly absorbing solutions. The light detectors covered the spectral region from 200 to 1200 mμ. Means were provided for shutting the detector during

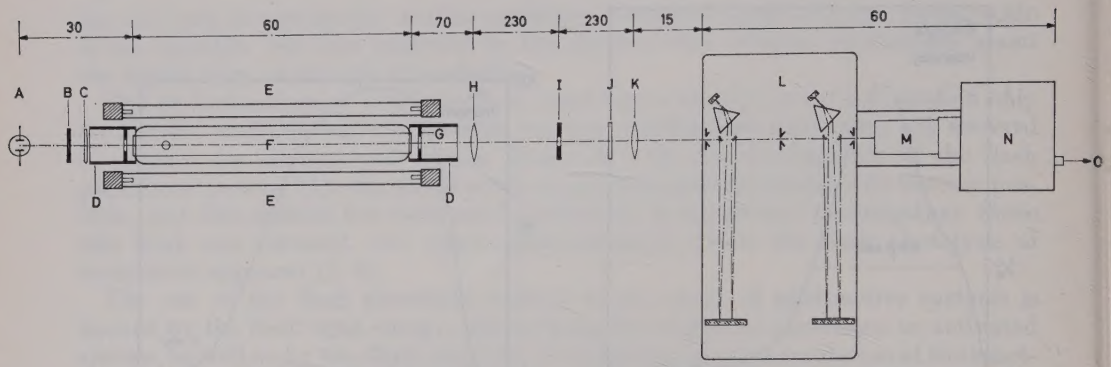


Fig. 3. The kinetic spectrophotometer.

A, xenon lamp (alternatively a tungsten lamp); *B*, electromechanical shutter; *C*, protective filter; *D*, light hoods; *E*, flash lamps; *F*, double-walled quartz cuvette, provided with end windows, filter solution in the outer cylinder; *G*, adjustable diaphragm; *H*, quartz lens (focal length 100 cm) imaging the light source on *I*, an adjustable diaphragm; *J*, neutral filter; *K*, quartz lens (focal length 15 cm) imaging the light source on the entrance slit of *L*, a double monochromator with interchangeable quartz and glass prisms (Zeiss MM 12); *M*, multiplier phototube (200–1200 mμ); *N*, phototube housing; *O*, connection to recording oscilloscope.

The Figure is not drawn to scale. Indicated dimensions in cm.

the flash, a feature which proved very valuable when luminescent solutions were investigated. The light detector was placed several meters from the flash lamps, thereby reducing stray light as well as magnetic disturbances of the electronic equipment during the discharge. For further suppression of stray light, the ends of the reaction cells were shielded with light hoods (D in Fig. 3). A narrow beam of the monitoring light traversing the cuvette was isolated by means of the adjustable diaphragm (G), set at an opening of about 7 mm. An image of the light source was thrown on a second adjustable diaphragm (I) by means of a condensing lens (H), focal length 100 cm. The function of this diaphragm was to isolate the light source image, thus reducing the stray light. A second condensing lens (K), focal length 15 cm, imaged the light source on the entrance slit of a monochromator; the size of the light source was reduced ten times. The optical system was so arranged that the monochromator prisms were filled with light. The light intensity could be reduced by introduction of gray filters (J) in the measuring beam. The dark signal was obtained by closing the shutter (B). A filter (C) was used to suppress possible photodecomposition caused by the measuring light source.

The strong vibration of the discharge tubes provoked by the discharge shock wave had a tendency to disturb the measurements, and it proved necessary to mount the monitoring light source and the cuvette separately on a heavy stone fundament.

Measuring light source.—A high pressure xenon lamp and a tungsten incandescent lamp were alternatively used as light sources. The xenon arc was preferred in view of its high intensity, resulting in a relatively lower level of flash stray light, and the tungsten lamp was chosen only when stability over longer periods was needed. The movement of the arc of the xenon lamp (Osram XBO 301, 15 V, 20 A dc, arc 1 × 1 mm) affected the light flux at the detector, and the resulting fluctuation, being negligible over time intervals of about 0.01 sec, became serious when measurements were

extended over a period of seconds. Current was supplied to the xenon lamp from a direct current generator (max. power 25 kW). The field magnets of the generator were fed with current from a high capacity storage battery and the motor was powered directly from the mains. The variations in line voltage affected the overdimensioned machine only at a slow rate. The ripple introduced by the commutators was bypassed by a 4000 μF condenser. A ballast resistance of 4 ohms was connected in series with the lamp.

The tungsten lamp was a krypton-filled sound film lamp (5.5 V, 0.75 A, Luma) with a straight tungsten thread. Current was supplied from a storage battery via a series resistance.

Monochromator.—The measuring light was dispersed in a double prism monochromator (Zeiss, Model MM 12, aperture f/10), with interchangeable glass and quartz prisms. A slit width of about 0.2 mm was used in most of the experiments, corresponding to a spectral band width (using the glass prisms) of 5 mμ at 800 mμ and 0.5 mμ at 400 mμ. The wavelength scale was checked against mercury emission lines.

Detector.—Multiplier phototubes were used as light detectors. A set of Du Mont and EMI tubes were available, with base connections and dimensions identical for easy interchangeability. The following Table indicates the types used, the spectral response, and the wavelength regions where each type was preferred.

Type		Spectral Response	Used in Spectral Region
EMI	9552 A	S–13	200–600 mμ
Du Mont	6292	S–11	400–600
Du Mont	K1305	S–10	600–700
EMI	9554	S–10	600–700
Du Mont	6911	S–1	> 700

The phototubes were of the end-on type, with semitransparent photocathodes, 44 mm in diameter, and equipped with ten dynodes. The Du Mont tubes were provided with an additional focusing shield controlling the phototube current. Whenever it was not necessary to take advantage of this possibility, the EMI tubes were preferred because of their better performance compared to the Du Mont tubes which showed a time lag effect, limiting their reliability. When these latter tubes were exposed suddenly to light of constant intensity, several seconds were required before the anode current reached equilibrium. This dynamic deviation from linear response was nearly independent of the light level and of the accelerating voltage. For an anode current of 1 μA, a change in current of 1 % was observed occurring over a time interval of 2 seconds. At 10 μA the drift amounted to 3 %. The EMI tubes on the other hand did not show any discernible effect of this kind, even at 10 μA.

It was assumed, apart from the time lag effect, that the phototubes used had linear response. It was noted that in the millisecond range no discrepancies in the experimental results arose when the various phototubes were interchanged, and this fact was considered as sufficient proof of the linearity.

The multiplier phototube, with associated electronics, indicated in Fig. 4, was contained in a separate housing and was mounted so as to permit easy replacement. The accelerating voltage was obtained from a stabilized high voltage supply (500–1200 V, 8 mA, ripple voltage 50 mV_{eff} at 1000 V), constructed at the Institute. Low-resistance wire wound bleeder resistors were used for voltage division except between

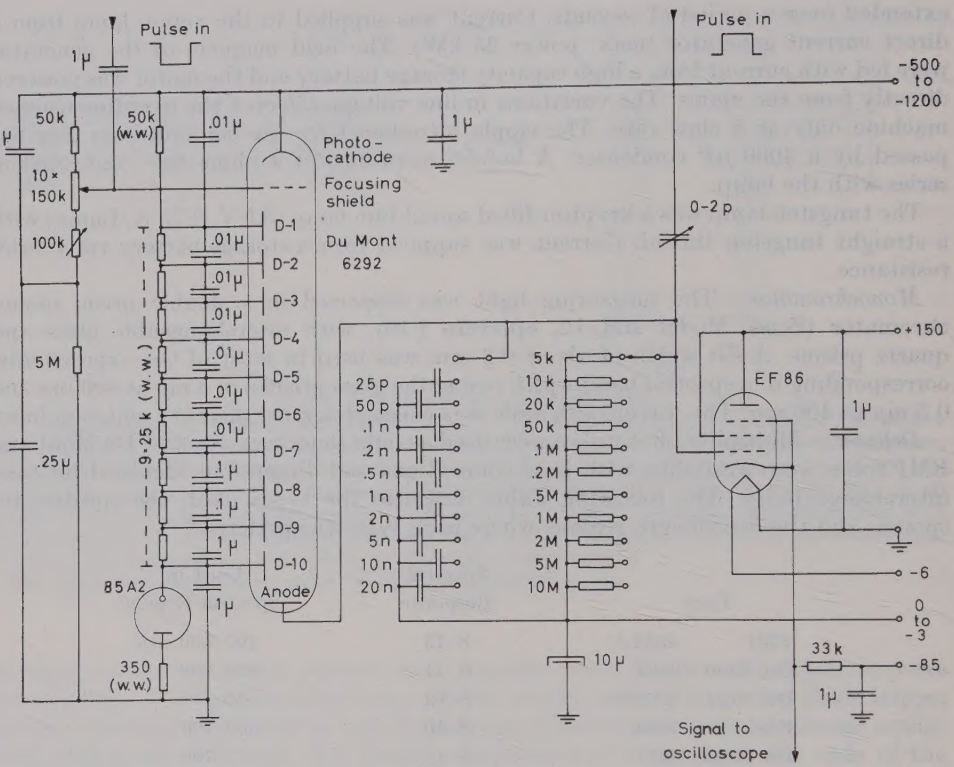


Fig. 4. Scheme of the multiplier phototube unit with associated electronics (cathode follower and shutting device) contained in the housing indicated *N* in Fig. 3. Resistances in ohms, capacities in farads.

the last dynode and the anode where a voltage stabilizing tube was inserted. Anode load resistances between 1000 ohms and 10 Megohms were used. It was possible to shunt the load resistance with capacities ranging from 25 pF to 10,000 pF, when it was convenient to integrate noise. The distributed capacity to the anode amounted to 25 pF. In most of the experiments a load resistance of 0.2 Megohm was used, yielding 5 μsec as the time constant of the detector. An overall voltage of 500–600 V was ordinarily applied across the phototube (refers to EMI tubes) and the anode current drawn was of the order of 1 μA.

The anode current was amplified in a cathode follower stage. The voltages for this circuit were derived from a separate stabilized supply. The filament of the cathode follower tube was heated with stabilized direct current. The cathode was direct-coupled to the oscilloscope amplifier and therefore it was necessary to maintain the cathode voltage near earth potential. This was achieved by connecting the phototube load resistance to a stabilized voltage between 0 and –3 V, set to the appropriate value by means of a potentiometric circuit, including a Beckman Helipot potentiometer (1000 ohms, 10 turns, 0.5 %). With this arrangement the signal resulting from exposure of the detector to light of constant intensity could be adjusted to zero,

thus allowing a high amplification of small changes in signal. By changing the compensating voltage the potentiometer scale was calibrated in terms of the C.R.T. beam deflections appearing on the oscilloscope screen.

Scattered light from the photolysis flash was not disturbing when non-luminescent solutions were studied. The highly luminescent fluorescein solutions, however, emitted strong light pulses which heavily overloaded the detector circuit. A device for shutting the detector was therefore constructed. The multiplier phototube was shut by driving the potential of the focusing shield (brought out to a separate pin in the Du Mont tubes) negative compared to that of the photocathode. The phototube current is only slightly affected by the shield potential as long as this is positive relative to the cathode, but abruptly decreases when the potential becomes negative. The shield potential was normally kept at the level yielding optimal phototube current. At the onset of the flash discharge the shield potential was, by application of a negative square pulse, automatically driven negative compared to that of the cathode for a short period. This results in shutting the phototube, but a disturbing signal is also introduced by capacitive coupling to the anode, as the capacity between the anode and screen leads inside the phototube is comparatively high, about 1 pF. The differentiated signal appearing at the anode, amounting to several volts, was therefore compensated by arranging a similar capacitive coupling with a positive square pulse of the same shape as the negative pulse. The coupling was effected by means of a variable condenser (0–2 pF) (see Fig. 4). The square pulses of opposite polarity were supplied from a square pulse generator with push-pull output (constructed at the Institute). Pulse rise time was 1 μsec and maximum pulse height 100 V. Pulse durations ranged from 20 μsec to 1 msec. It was possible to suppress the stray light signal from several tens of volts down to about 0.1 V with this device.

Oscilloscope recording.—The detector signals were fed to an oscilloscope, Tektronix Model 533, equipped with a dc preamplifier, Model 53/54 B. Most of the experiments were carried out at an amplification of 50 mV per cm deflection on the screen. The linearity of the C.R.T. horizontal and vertical axes was tested and found to be very good within the central 4×8 cm area of the screen. The time base was calibrated, at intervals, by means of a crystal-controlled time mark generator, Tektronix Model 181.

Changes in detector signal occurring during a single sweep were recorded. The sweep triggering pulse was obtained from the high voltage pulse used for the initiation of the flash. The discharge tubes glow for some tens of microseconds before the heavy discharge builds up, allowing the sweep to trace the signal immediately preceding the flash. In order to render this reference signal visible also at slower sweep rates, the period between the triggering of the sweep and the build-up of the flash was increased by means of a time relay controlling the triggering pulses. The trace was photographed and an enlarged image of the negative was reproduced graphically on millimeter paper.

Cuvettes.—Cylindrical quartz cuvettes of two different lengths, 20 cm and 60 cm, were used. The inner diameter of the longer cuvette was 1.1 cm, the shorter 1.6 cm. The cuvettes were provided with optically flat end windows and with an outer mantle containing a liquid filter. The filter thickness was 1 cm. It was important to avoid temperature gradients in the cuvettes, in view of the long optical paths. The filter liquid was, to this end, circulated through a thermostat by means of an all-glass centrifugal pump.

Chapter 2. Experimental

Chemicals

Fluorescein, Merck's, was submitted to a chromatographic purification. An adsorbing column consisting of a mixture of activated aluminium oxide and talcum (weight ratio 1 to 3) was used, as recommended by Koch [5] for the separation of fluorescein from its derivatives. The fluorescein was dissolved in an alkaline water solution (0.01 *M* NaOH). After adsorption the dye was recovered by destroying the column and shaking the coloured part of the adsorbent with excess 0.01 *M* NaOH. The dissolved fluorescein was precipitated by addition of acetic acid, the precipitate filtered and washed with water. After drying at 100°C overnight, the pure fluorescein, being somewhat hygroscopic, was stored in a desiccator.

The purity of the fluorescein was judged from absorption measurements. The molar extinction coefficient at the peak, 491 mμ, was found to be 8.8×10^4 for a 5 μM solution of fluorescein in 0.01 *M* NaOH. No correction was made for the effect of the fluorescent light.

The corresponding values reported by other authors invariably lie considerably lower. Imamura [6] in a recent paper surveys earlier spectrophotometric measurements and states 7.8×10^4 as the extinction coefficient at the peak after due correction for the influence of the fluorescent light. No measurements on chromatographically purified fluorescein are reported and the high value found in this investigation is attributed to the high efficiency of this method of purification compared to commonly used precipitation or recrystallization procedures.

p-Phenylenediamine, c.p., was purified by twofold sublimation under a high vacuum and was kept in an oxygen-free atmosphere.

p-Dioxane, c.p., was boiled with sodium for twelve hours and submitted to a fractional distillation. The distillate was further purified by a threefold freezing procedure. Throughout the process water was very carefully excluded, and the pure product was kept in a dry argon atmosphere.

Perchloric acid, 70 %, anal. grade, was distilled at reduced pressure in order to remove any chlorine.

Water. Doubly distilled water was used.

Other chemicals were of reagent grade.

Preparation of solutions

The solutions were prepared by diluting weighed amounts of a fluorescein stock solution with the required buffer. Citric acid-citrate buffers were used in the pH range 2 to 6, phosphate buffers between pH 6 and 8, and boric acid-sodium hydroxide buffers between pH 8 and 11. The buffer concentration was 0.01 *M*. Perchloric acid and sodium hydroxide were used for the establishment of pH at the low and high ends of the pH scale, respectively.

Other solutes also were added on a weight basis. Solutions containing *p*-phenylenediamine were prepared immediately before use.

For each experiment the absorption spectrum and the pH of the solutions were measured.

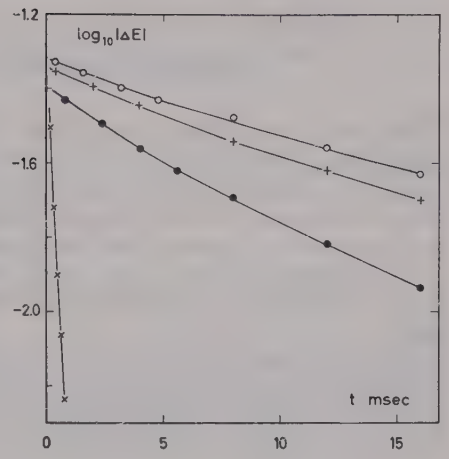


Fig. 5. Oxygen effect on the decay of the fluorescein triplet at different stages of the degassing procedure. × Freezing, evacuating and melting three times; ● the same procedure, followed by flushing with argon once and three further cycles of degassing; + the previous procedure followed by an additional flushing with argon and three further cycles of degassing, with a low argon pressure applied above the deaerated solution; ○ the same, but no argon above the solution. The logarithm of the decrease in extinction at 491 mμ is plotted against the time after flashing. Fluorescein concentration 0.010 μ*M* (in 0.01 *M* NaOH). Cuvette length 60 cm.

Deaerating of solutions.—The bulk of the experiments were carried out with deaerated solutions. Careful degassing was most important in view of the generally strong quenching effect of oxygen on molecules in the triplet state. Preliminary experiments revealed that even after repeated freezing and evacuating, traces of air remained in the solutions, affecting the kinetics. Therefore, flushing with an inert gas was combined with this procedure. The technique adopted was to freeze, evacuate and melt three times and then to shake the solution in the presence of argon. This entire procedure was carried out three times, the shaking with argon being excluded the last time. Dry ice was used for cooling. The degassing was carried out in a flask connected to a mercury diffusion pump with a liquid air trap. The flask was connected to the cuvette via ground glass joints, and arrangements were made so that the flask contents could be transferred into the cuvette without coming into contact with the lubricated joints. Once the cuvette was filled, a low argon pressure was applied above the solution to suppress formation of water vapor bubbles. Cuvette and flask were removed from the vacuum line after closing a stopcock.

Argon for aluminum welding (AGA, Stockholm) was used as flushing gas.

The concentration of the oxygen remaining after the degassing was estimated to be lower than 10^{-8} *M*. This value was calculated under the assumption that oxygen alone was responsible for the decay of the fluorescein triplet, with a collision efficiency of unity. The true oxygen content probably was even lower. The oxygen effect on the triplet decay at different stages of the degassing procedure is demonstrated in Fig. 5. It is judged from the decay curves that after nine cycles of freezing and degassing no further oxygen will be removed. The argon above the solution did not affect the kinetics, as revealed by comparison with an experiment omitting the inert gas.

Spectrophotometric measurements.—A Zeiss non-recording spectrophotometer, PMQ II, with quartz optics was used for absorption measurements. Quartz cuvettes of thickness 1 and 5 cm were used. The slit widths corresponded to a spectral band width of 0.4 mμ (at 400 mμ) or less.

pH measurements.—pH was measured with a Radiometer pH meter, Type PHM3b, provided with a glass electrode and a saturated calomel electrode as reference.

Experimental conditions for the kinetic investigation

The kinetic experiments were, except where otherwise stated, performed using a discharge energy of 6000 J (400 μF, 5.5 kV).

The time resolution of the kinetic measurements was principally determined by the flash duration. If the primarily formed photoproducts decay more rapidly than the flash light intensity, their occurrence would be readily overlooked. Judging from the intensity-versus-time curve shown in Fig. 2 (corresponding to a 6000 J flash), intermediary products of lifetimes shorter than about 20 μsec accordingly might escape observation.

The absorption measurements in the wavelength region of fluorescein luminescence were obscured by light re-emitted during the flash, and it was in these cases necessary to shut the detector during flashing. The extinction changes recorded after opening the detector were extrapolated back to the moment of onset of the dark reactions. The photoexcitation was assumed, somewhat arbitrarily, to be completed 70 μsec after the build-up of the discharge (at the energy of 6000 J), and this time was chosen as the moment of onset of the dark reactions. It should be pointed out that the zero time in the representation of the experimental data refers to the moment of build-up of the discharge.

Only the visible part of the flash light was utilized for excitation. The transmittance of the filter isolating the visible spectrum is shown in Fig. 2.

The fluorescein solutions were deaerated, except where otherwise stated.

All experiments were carried out at 25°C.

A series of about 300 experiments were performed. A fresh solution was prepared for each experiment. The solutions were flashed several times. The permanent photoproducts apparently did not affect the kinetics but the data reported are, nevertheless, in most cases taken from kinetic measurements at the first flash.

The kinetic data reported refer to isolated experiments but are in all cases supported by supplementary experiments. The reproducibility of the absorption measurements was shown to be high and the limit of reliability of the results probably was set by systematic errors.

Evaluation of oscillograms.—The changes in light transmission of the illuminated solutions were measured in terms of vertical deflections on the oscilloscope screen. The detector response was assumed to be linear. The changes in concentration of the reacting species were related to the deflections by means of Beer's law.

In the case of i different species, appearing in concentrations c_i^0 at the moment of build-up of the flash discharge ($t=0$), the difference between the extinctions at time t and time 0, ΔE, is obtained as

$$\Delta E = \log_{10}\frac{I_0}{I_t} = \log_{10}\frac{d_0}{d_t} = b\sum \varepsilon_i\,(c_i^t - c_i^0) \qquad \{1\}$$

where ε_i is the molar extinction coefficient of species i, c_i^t the concentration (in M) at time t after the build-up of the flash and b the optical path in the cuvette (in cm). I_0 and I_t represent the intensities of transmitted light at times 0 and t respectively, d_0 and d_t the corresponding, recorded deflections.

A case, frequently encountered, is the formation of i species starting with one species 0 (one molecule formed for each molecule 0 consumed). The extinction change in this case becomes

$$\Delta E = b \sum (\varepsilon_i - \varepsilon_0) c_i^t. \qquad \{2\}$$

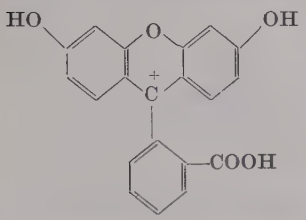
When $\varepsilon_i = 0$, one obtains

$$\Delta E = -b \varepsilon_0 \sum c_i^t. \qquad \{3\}$$

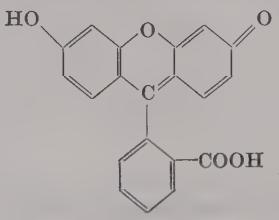
Chapter 3. Ionic and molecular forms of fluorescein in water solution

Fluorescein exists in five different monomeric forms in water solution in the pH range 0–13. The dissociation stages have recently been studied by Zanker and Peter [7] who determined the corresponding dissociation constants in dioxane-water solutions. The use of their data in the present investigation is precluded, however, by the differences in solvent character and ionic strength. Other studies reported are of more qualitative nature.

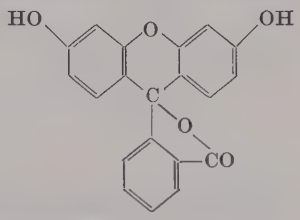
Adopting the scheme advanced by Zanker and Peter the ionic and molecular forms of fluorescein may be described as follows (the mesomerism is not indicated).



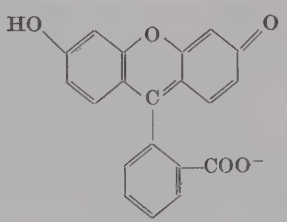
Cation.



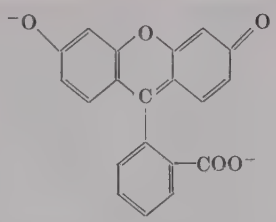
Neutral molecule.
Quinonoid form.



Neutral molecule.
Lactonic form.



Mono-anion.



Di-anion.

The lactonic form is present in organic, non-ionic solvents. Fig. 6 shows the absorption spectrum of chromatographically pure fluorescein dissolved in carefully dried dioxane. The absorption in the visible region is very low ($\varepsilon_{max} = 5$ at 450 mμ) and probably should be attributed to a quinonoid form of the dye. Zanker and Peter report a 20 times stronger absorption in the visible, the discrepancy most likely being explained by differences in water content.

The dissociation steps were studied by absorption measurements at different acidities. A stock solution containing fluorescein and sodium hydroxide, citric, phos-

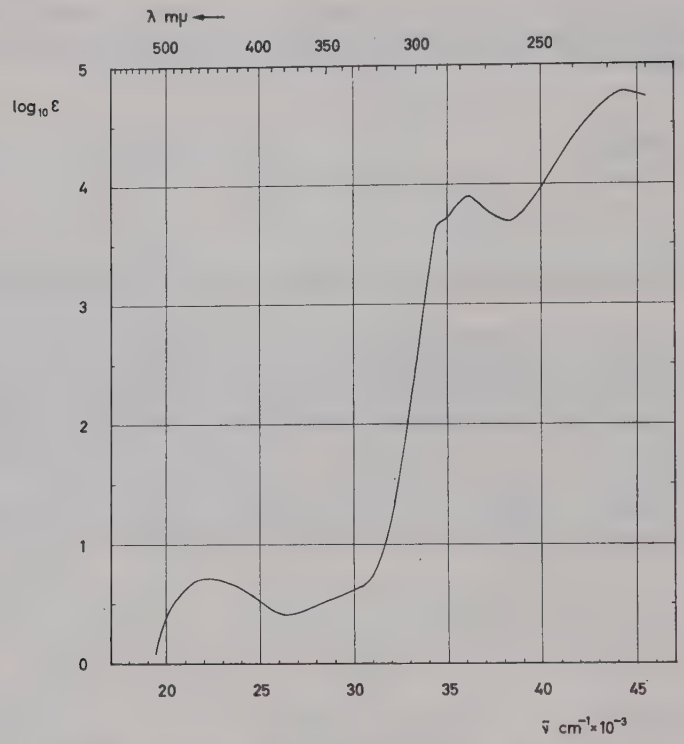


Fig. 6. Absorption spectrum of chromatographically pure fluorescein in dry dioxane.

phoric, and boric acids was prepared. The acidity was varied by adding appropriate amounts of hydrochloric acid to measured volumes of the stock solution and then diluting with water to a specified volume. The concentration of fluorescein was 10 μM, and the ionic strength of the buffer solution approximately 0.01 (at the highest acidities this value was exceeded). Absorption measurements were made in the pH range 1.3 to 8.8, at intervals of 0.3. The molar extinction coefficients are traced against pH in Fig. 7 at three characteristic wavelengths. Three dissociation equilibria are indicated by the shape of the curves. In strongly acidic solution the cation is present. This dissociates at about pH 2 to the neutral forms. Between pH 4 and 5 the mono-anionic form appears, which in turn dissociates at about pH 7 yielding the di-anion.

The dissociation constants were obtained using a graphical method commonly employed when dissociation equilibria between coloured conjugates are concerned. If the concentrations of an acid and its conjugate base are expressed, at a certain wavelength, in terms of the extinction coefficient of the acid (ε_a), the base (ε_b) and the extinction coefficient (ε) of the system in equilibrium, the following expression is obtained

$$\mathrm{p}K_a = \mathrm{pH} - \log_{10}\frac{\varepsilon - \varepsilon_a}{\varepsilon_b - \varepsilon} \qquad \{1\}$$

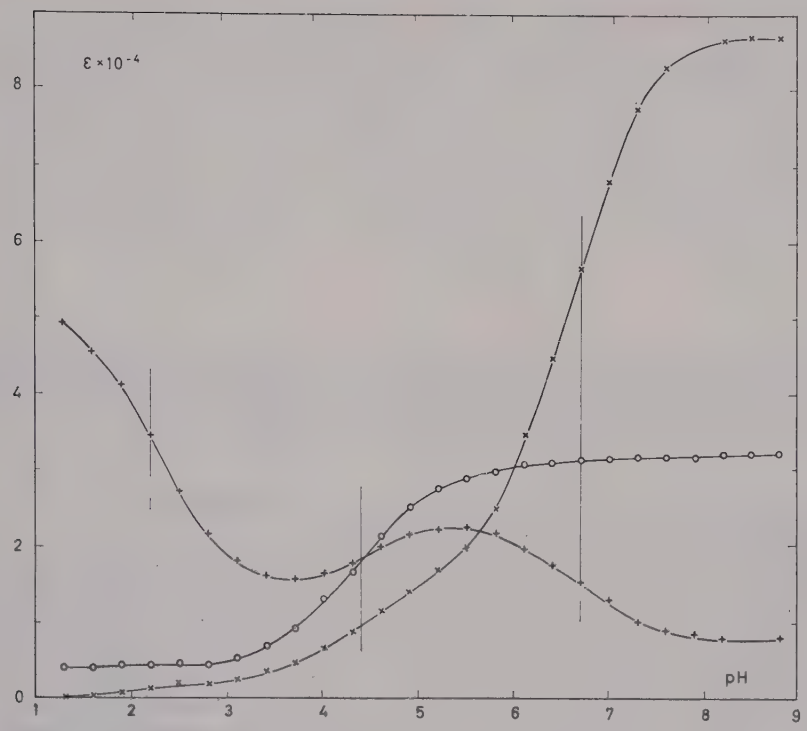


Fig. 7. The pH dependence of the absorption spectrum of fluorescein in water solution, measured at three characteristic wavelengths, + 437 mμ, ○ 464 and × 491 mμ. Fluorescein concentration 10 μM, optical path 1 cm.

where K_a is the acid dissociation constant at the actual ionic strength. If the expression $\log_{10}(\varepsilon - \varepsilon_a)/(\varepsilon_b - \varepsilon)$ is plotted against pH, the intersection of the resulting curve with the pH axis will give the pK_a value. This method of evaluating the dissociation constant can be used only if the extinction coefficients of the acid and base are known. The different dissociation stages of fluorescein lie comparatively close together, and only the cationic and di-anionic forms can be isolated. Fig. 7 shows, however, that at the wavelength 464 mμ, there is an approximate equality between the extinction coefficients of the cation and the neutral molecule on the one hand, and of the mono- and di-anions on the other hand. If these extinction coefficients are used in the calculation, an apparent pK_a value of 4.4 (this value may in the present case be approximated to the true pK_a value) is found for the dissociation of the neutral molecule into the anionic form, as shown in Fig. 8. The dissociation of the mono-anion into the di-anion is most conveniently followed at the wavelength 491 mμ, corresponding to the strong absorption peak of the di-anion. Fig. 8 gives a value of 6.7 for the corresponding pK_a. The equilibrium between the cation and the neutral molecule was studied at 437 mμ, corresponding to the absorption peak of the cationic form. At this wavelength the mono-anion has considerable absorption, and a correction was introduced for the appearance of this form at pH higher than 3. The curve

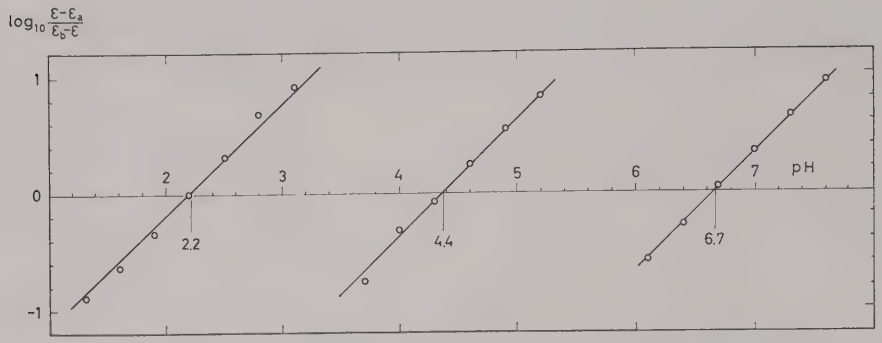


Fig. 8. Graphical determination of the pK_a values of the dissociation equilibria of fluorescein, according to Eq. {1}, from absorption data presented in Fig. 7. pK_a for the fluorescein cation is 2.2, for the neutral molecule 4.4 and for the mono-anion 6.7. Straight lines with slope 1 are drawn through the different sets of points.

obtained after correction indicates a pK_a value of 2.2. Throughout the Figure, straight lines with slope one are drawn, as required by Eq. {1.}

The different forms of fluorescein were further characterized by measurements of their absorption spectra. The absorption of the cationic form was measured in 1 *M* sulphuric acid, the di-anion in 0.01 *M* sodium hydroxide solution. The two remaining forms were measured at pH 3.3 and 5.5, that is, at the acidities where each of them predominates. No correction was applied for the existence of the neighbouring forms at these acidities. The spectra obtained are shown in Fig. 9. In the following Table the absorption peaks in the visible, and the corresponding extinction coefficients, are reported.

pH	Absorption peaks cm^{-1}	$m\mu$	$\varepsilon \times 10^{-4}$	Predominant form
0	22,850	437	5.5	Cation
3.3	22,850	437	1.6	Neutral molecule
5.5	22,000	455	3.0	Mono-anion
	21,100	475	3.1	
12	20,400	491	8.8	Di-anion

The absorption in the visible, measured at the pH where the neutral molecule predominates, although low, cannot be attributed exclusively to the occurrence of cations and mono-anions. It seems therefore that the neutral form does not appear entirely in the lactonic state which is uncoloured, but also to a certain extent in the quinonoid form. It has been stated by Scharf [8] that fresh solutions in this pH region contain the quinonoid form, part of which, however, slowly converts to the lactonic form. As a slow attainment of equilibrium would introduce a serious complication in the flash photolysis experiments, it was deemed necessary to explore this possibility. A 10 μM fluorescein solution was prepared by adding an alkaline fluorescein solution to a buffer solution of pH 3.3. The absorption peak at 437 $m\mu$ was measured immediately and after a month; no decrease in extinction was observed. The flash photolysis investigation, carried out at this pH also indicated a very rapid establishment of equilibrium.

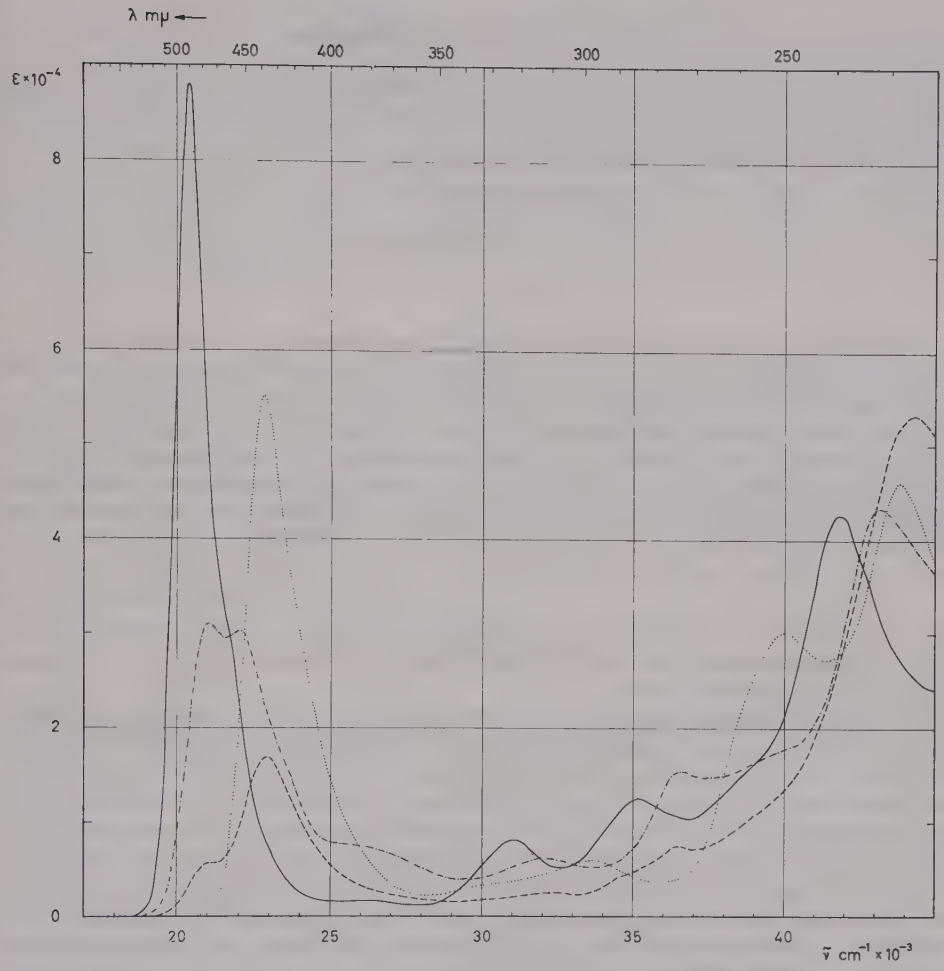


Fig. 9. Absorption spectra of fluorescein in water solution, at the acidities of predominance of the cation, 1 *M* H_2SO_4 · · · ·; the neutral molecule, pH 3.3 ——; the mono-anion, pH 5.5 —·—·—·— and the di-anion, 0.01 *M* NaOH ———. Fluorescein concentration 10 μM. Optical paths 1 and 5 cm.

So far only the monomeric forms of fluorescein have been considered. The dye is, however, apt to dimerize and it proved necessary to study the possibility of dimer formation at the concentrations used in the photochemical investigation (10 μM or less). The dimerization of the di-anion has been studied [9], and the results show that at low concentrations dimerization is negligible. For the other forms no data have been found in the literature. Measurements of the concentration dependence of the absorption spectra at pH 1, 3.3 and 5.5 therefore were carried out. The results showed that Beer's law was valid in the concentration range 1–10 μM and the possibility of dimer formation consequently may be discounted.

Chapter 4. Kinetic investigation

Fluorescein, by absorption of visible light, is raised to the first excited singlet state (S^*) from which it may return to the ground state (S) by re-emission of the absorbed energy, or by a radiationless transition,

$$S + h\nu_{\text{visible}} \rightarrow S^*$$

$$S^* \rightarrow S + h\nu_{\text{fluorescence}}$$

$$S^* \rightarrow S.$$

The high intrinsic reactivity of the excited singlet state is counteracted by its very short lifetime (4.5×10^{-9} sec for the di-anion in water solution [10]) and this state is not ordinarily responsible for the photochemical activity of the dye. There is, however, much evidence accumulated for the existence of a more long-lived excited state of lower energy, which is assumed to be the lowest triplet state (T). The triplet level of fluorescein is not reached by direct photoexcitation, the singlet-triplet absorption being too low, but is populated exclusively by a transition from the excited singlet state,

$$S^* \rightarrow T.$$

The comparatively long lifetime of the triplet state is due to the forbiddenness of the triplet-singlet transition, while its high reactivity is associated with the biradical nature of the electronic state.

The present investigation is concerned with the study of the triplet state of fluorescein and of the mechanisms responsible for its action in photoreductions and photo-oxidations. Before proceeding to the investigation of more complex reaction systems the reactions of the triplet in pure water solution were studied. Several unexpected features were revealed which were believed to be of importance for the understanding of the photosensitizing action of the dye, and the phenomena observed were subjected to a detailed analysis. The results obtained were illustrated and verified in the study of a few typical photoreduction and photo-oxidation reactions. The inhibitory effect, excerted by certain substances on photosensitized reactions, was also investigated (included in the Photoreduction Section).

Before proceeding to the treatment of the photochemical reactions proper, a short discussion of ionization reactions will be presented. The ionic equilibrium of the solutions studied is disturbed during flashing and the reactions leading to re-establishment of equilibrium may under certain circumstances introduce difficulties in the interpretation of the kinetic data. It was therefore necessary to take these reactions into account.

Ionization reactions

The ionic equilibrium of a photoactive system, like fluorescein in water, is changed on illumination, due to the modified acid-base character of the excited species. Ionization reactions will in many cases have time to occur during the short lifetime of the excited state [11]. Weller [12] has quantitatively studied the effect of such ionization reactions on the fluorescence of a series of fluorescent compounds. Other intermediary

or stable products appearing during a photochemical reaction will also not be formed under conditions of equilibrium and consequently will undergo ionization reactions. The rates of these reactions depend upon the acid-base strength of the compounds in question and upon the possibility of proton exchange with the solvent.

In the present photochemical study the occurrence of ionization reactions introduces a complication in the analysis of the kinetics of the photoreactions. This complication can be eliminated by arranging conditions allowing establishment of ionic equilibrium within the time of resolution of the absorption measurements. The necessary requirements for rapid establishment of equilibrium are discussed in this section.

An acid-base equilibrium

$$HA + H_2O \underset{k_2}{\overset{k_1}{\rightleftarrows}} A^- + H_3O^+$$

with an acid dissociation constant K_a, may be considered to be the consequence of two opposing reactions, one corresponding to a dissociation (rate constant k_1) and the other to an association (rate constant k_2). In most cases the bimolecular association reaction, having a very low activation energy, is mainly diffusion controlled. Weller has experimentally found rate constants of the order of 10^{10} M^{-1} sec^{-1} for a series of organic acids. The rate constant of the dissociation resulting from reaction with water can be expressed as

$$k_1 = K_a k_2 \approx K_a \times 10^{10} \text{ sec}^{-1}.$$

The mechanisms considered above will be predominant in acid solutions. In alkaline solutions the equilibrium is more conveniently written as

$$HA + OH^- \underset{k_2'}{\overset{k_1'}{\rightleftarrows}} A^- + H_2O.$$

In this case the dissociation reaction (rate constant k_1') is diffusion controlled. The order of magnitude of k_1' is the same as of k_2, i.e. $k_1' \approx 10^{10}$ M^{-1} sec^{-1} (k_2 is somewhat higher than k_1', due to the higher mobility of the proton compared to that of the hydroxyl ion). The rate constant for formation of acid by reaction of the base A^- with water can be formulated as

$$k_2' = K_b k_1' \approx K_b \times 10^{10} \text{ sec}^{-1}$$

where K_b represents the base constant.

In the case of reactions between charged species, coulombic interaction must be taken into account. The rate constant for formation of, for example, neutral fluorescein from the cation and a hydronium ion is decreased by a factor of about 2 by the effect of electrostatic repulsion. (This value was calculated from a formula put forward by Debye [13].) This decrease will be neglected in the present semi-quantitative treatment.

The actual flash photolysis experiments were carried out with a time resolution of about 10^{-5} sec, and the equilibrium should be established within this time. It is

apparent from the values of the rate constants shown above that this requirement is fulfilled only when

$$pK_a < 5 \text{ or } pH > 9, \text{ acid initially present in excess, or}$$
$$pK_a > 9 \text{ or } pH < 5, \text{ base initially present in excess.}$$

The sluggishness of the ionization reactions near the neutral point is a consequence of the low concentration of hydronium and hydroxyl ions. The proton exchange may be facilitated, however, by adding a second acid-base system. An equilibrium involving this second system (HB–B$^-$) is developed,

$$HA + B^- \underset{k_2''}{\overset{k_1''}{\rightleftarrows}} A^- + HB$$

with the equilibrium constant $K = \frac{k_1''}{k_2''} = \frac{K_a}{K_a''}$, K_a'' being the acid dissociation constant of HB.

Weller experimentally determined the rate constants for reactions between naphtols and a series of bases, and found k_1'' to be of the order of $10^9\ M^{-1}\ \text{sec}^{-1}$ when K_a was approximately equal to or greater than K_a''. Using these results the rate (v_1'') of formation of the base A$^-$ becomes, when $K \geqslant 1$

$$v_1'' = c_{HA}\, c_{B^-}\, k_1'' \approx c_{HA}\, c_{B^-} \times 10^9\ M\ \text{sec}^{-1}.$$

The relative rate (v_2'') of formation of the acid HA can be written as

$$v_2'' = c_{HB}\, c_{A^-}\, k_2'' \approx \frac{c_{HB}\, c_{A^-}}{K} \times 10^9\ M\ \text{sec}^{-1}.$$

If the system HB–B$^-$ is added as buffer, at its optimal buffer capacity, and alone controls the acidity of the solution, then the pH will be equal to pK_a''. This special case will be considered in the following. Considering only the formation of acid HA from excess base, it is easily seen that in the pH range 6–8 the most unfavourable situation from the point of view of rapid establishment of equilibrium would be that $pK_a = 6$ and $pH = 8$ ($= pK_a''$). In this case K will be large and the rate v_2'' accordingly low,

$$v_2'' = c_{HB}\, c_{A^-} \times 10^7\ M\ \text{sec}^{-1}.$$

It is seen that the base A$^-$ will disappear with the required rate, $10^5\ \text{sec}^{-1}$, at a buffer concentration of $10^{-2}\ M$. An analogous result is obtained if acid initially is present in excess.

The results of the above discussion were considered when the experimental investigation was undertaken. A buffer concentration of $10^{-2}\ M$ was used in most of the experiments. Higher concentrations, although preferable from the point of view of rapid establishment of equilibrium, were avoided due to the increasing effect of the ionic strength and in order to keep contamination of impurities as low as possible.

Photoreactions in deaerated water solution

When fluorescein in oxygen-free water solution is illuminated by visible light of moderate intensity, no irreversible changes are observed, even after prolonged exposures [14]. This implies that the triplet molecules present during the excitation are deactivated without producing any net reaction. The direct study of the decay of the triplet by the flash photolysis method revealed that even in the absence of substrates the triplet initiates a series of complex reactions. These reactions are, however, of a reversible character, thus accounting for the light stability of the dye.

The kinetic measurements showed that only one primary photoproduct, assumed to be fluorescein in the triplet state, was present during the flash. It proved possible, with the high light energies available, to convert the dye quantitatively to this state, that is, the solutions were light saturated. The recovery of ground-state fluorescein occurred in three characteristic reaction stages. During the rapid first stage the triplet disappeared. The decay of the triplet was not associated with a quantitative recovery of the ground-state fluorescein, but instead, about half of the triplet molecules were converted to two further intermediary products which were characterized by their strong absorption bands. Kinetic experiments revealed that these intermediates were produced by collisions between two triplet molecules or between one triplet and one ground-state molecule, and an electron transfer mechanism was assumed to be responsible for the reaction, yielding a semi-reduced and a semi-oxidized radical form of fluorescein. In the second, slower stage of the reaction the semi-oxidized radicals disappeared. Their depletion was paralleled by an increase in concentration of ground-state fluorescein, but again the decay and recovery were not balanced. The two radical forms reacted to some extent with each other to restore fluorescein, while about one half of the semi-oxidized radicals apparently underwent a dimerization reaction. When the semi-oxidized form was exhausted, the rate of decay of the semi-reduced molecules left behind became very slow, forming the third stage of the reaction. The ultimate recovery of ground-state fluorescein was complete only after a period of seconds. A slight permanent change was observed (4 % at 6000 J).

In the following sections the experimental results will be treated in detail.

The triplet state

The first intermediary product appearing after photoexcitation was characterized by a weak, diffuse absorption in the visible and near infrared region of the spectrum. This absorption was assigned to the lowest triplet state of fluorescein.

If complete conversion to the triplet state could be achieved during flashing, the starting conditions for the dark reactions would be known, greatly facilitating the kinetic analysis. A study of the light energy dependence of the conversion therefore was undertaken.

Fig. 10 shows results obtained for a 0.5 μM fluorescein solution in 0.01 M NaOH. The extinction change at 600 mμ during flashing was chosen as a measure of the conversion to the triplet state. Flashes of an electrical energy of 12,000 J (800 μF, 5.5 kV) were used and the light incident on the solution was varied by means of screens. It is apparent from the Figure that light saturation was achieved. The decrease in initial conversion at the highest light energies probably is due to a slight photodecomposition of the triplet molecules.

A higher flash light energy, although preferable from the point of view of excitation,

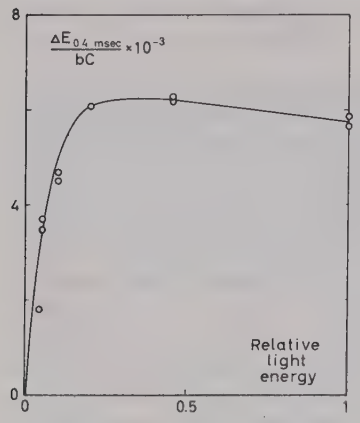


Fig. 10. The degree of conversion of fluorescein to the triplet state, as a function of the light energy. The extinction coefficient at 600 mμ, measured 0.4 msec after flashing, is plotted against the light energy incident on the solution. The electrical energy was 12,000 J, and the light energy was varied by means of screens. Fluorescein concentration 0.5 μM (in 0.01 M NaOH), cuvette length 60 cm.

implies a longer flash duration and as a compromise, an electrical energy of 6000 J (400 μF, 5.5 kV) was used in most of the experiments. It was shown that at this energy complete conversion was reached at fluorescein concentrations up to about 1 μM, while at higher concentrations the rate of triplet decay became so fast that only partial conversion was achieved.

Spectra.—The triplet absorption was measured at different acidities. In the ultraviolet (the lowest wavelength studied was 280 mμ) the triplet absorption did not differ in a significant way from that of the ground-state singlet. The triplet absorption was experimentally measured as the difference $(\varepsilon_T - \varepsilon_S)$, and the accuracy of the determination of ε_T consequently was decreased in the wavelength region of the strong absorption band of fluorescein in the visible. Therefore, only the absorption on the low-frequency side of the ground-state absorption band was studied in detail.

The absorption spectra of the triplet at the acidities where each of the different ionic and molecular forms of fluorescein predominates, are shown in Fig. 11. The shape of the triplet spectrum is not affected by pH changes between pH 0 and 5.5, although the absorption intensity varies slightly. It cannot be stated with certainty whether the differences are real or due to slight variations in the degree of conversion. The triplet spectrum in alkaline solution is, on the other hand, quite distinct from the spectrum in acid solution. Apparently an absorption peak is situated beyond 950 mμ. Measurements at higher wavelengths were, however, precluded by the increasing absorption of water.

It was expected that the modified electron structure of fluorescein in the triplet state would affect the dissociation equilibria. In a series of experiments the change from acid to base triplet was studied in order to establish the corresponding dissociation constant. The pH was varied between 5.8 and 7.8 in steps of 0.3, and the absorption changes at 570 mμ were measured during flashing. The results are shown in Fig. 12. By plotting $\log_{10}(\varepsilon - \varepsilon_a)/(\varepsilon_b - \varepsilon)$ against pH (see Eq. {3.1}) the pK_a value was evaluated to be 7.0, that is, nearly equal to that of the mono-anion in the ground state. It is inferred from this result that the two triplet forms existing in neutral solution are the mono- and di-anions.

The cation, neutral molecule and mono-anion in the triplet state apparently had very similar absorption and it was not attempted to measure the corresponding dissociation constants.

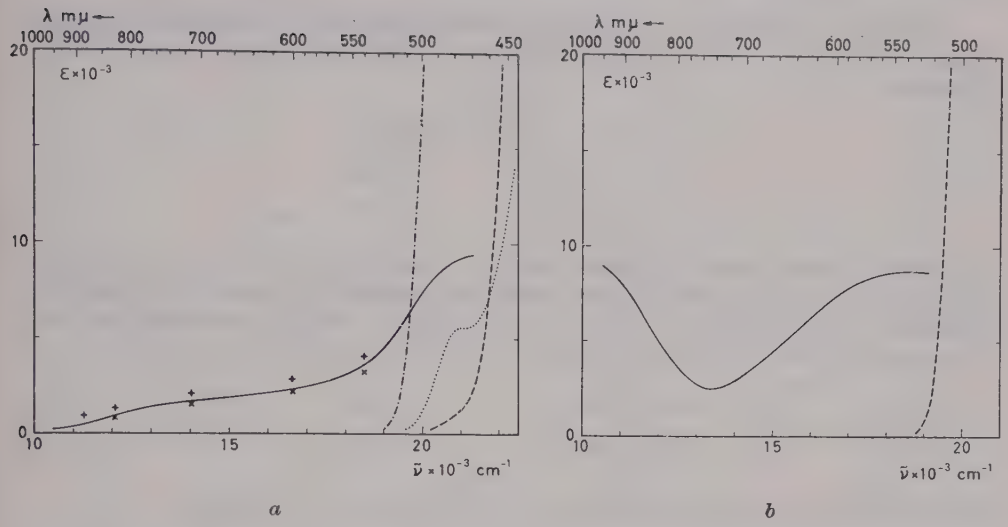


Fig. 11. The absorption spectrum of the triplet state of fluorescein as it appears during flashing. Fluorescein concentration 0.5 μM, cuvette length 60 cm.

(*a*) Triplet spectrum in acid solution, —— pH 1.7, × pH 3.3 and + pH 5.5. The spectra of fluorescein at the corresponding acidities are drawn for comparison; – – – – pH 1.7, · · · · pH 3.3 and –·–·–·– pH 5.5. (*b*) Triplet spectrum in alkaline solution, —— pH 12. The spectrum of fluorescein at the corresponding pH is drawn for comparison – – – –.

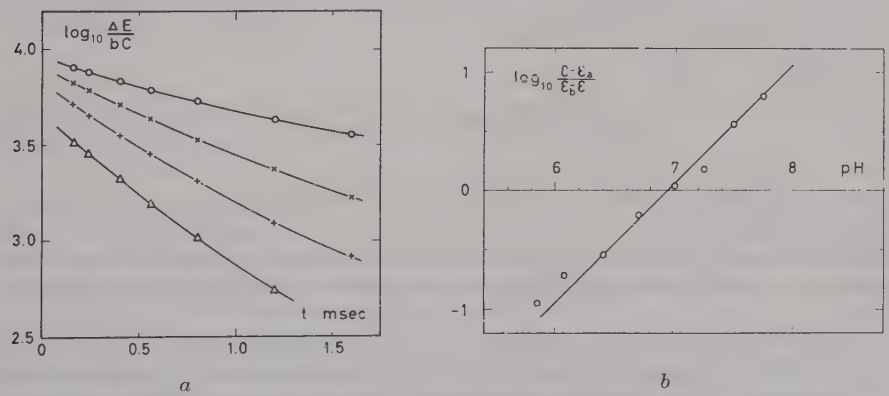


Fig. 12. The dissociation equilibrium of the triplet state of fluorescein. Fluorescein concentration 0.5 μM, cuvette length 60 cm.

(*a*) Transient extinctions at 570 mμ, measured at different acidities; ○ pH 12, × pH 7.50, + pH 7.00, △ pH 5.85. (*b*) Graphical determination of the pK_a value of the triplet, according to Eq. {3.1}. A straight line with slope 1 is drawn through the points. pK_a = 7.0.

Triplet decay.—The decay of the triplet state was studied at concentrations ranging from 0.01 μM to 4 μM. The following reactions were expected to be responsible for the decay,

$$T \rightarrow S \qquad \text{(rate const. } k_1\text{)} \tag{1}$$

$$T + T \rightarrow T + S \qquad (k_2) \tag{2}$$

$$T + S \rightarrow S + S \qquad (k_3). \tag{3}$$

The kinetic data showed, however, that S did not reappear at the same rate as T disappeared, and the existence of two additional intermediary products of fluorescein (designated by the letters R and X) was established (see the succeeding section). These products are formed in equimolecular amounts, according to the processes

$$T + T \rightarrow R + X \qquad (k_4) \tag{4}$$

$$T + S \rightarrow R + X \qquad (k_5). \tag{5}$$

Quenching of the triplet state by R and X also had to be considered,

$$T + X \rightarrow S + X \qquad (k_6) \tag{6}$$

$$T + R \rightarrow S + R \qquad (k_7). \tag{7}$$

The rate of disappearance of T then may be expressed as

$$-\frac{dc_T}{dt} = k_1 c_T + (k_2 + 2k_4) c_T^2 + (k_3 + k_5)\, c_T c_S + k_6 c_T c_X + k_7 c_T c_R \qquad \{1\}$$

where c_i is the concentration of species i.

The initial rate of decay is

$$-\left[\frac{dc_T}{dt}\right]_0 = k_1 C + (k_2 + 2k_4) C^2 \qquad \{2\}$$

if complete conversion to the triplet state is achieved during flashing (C is the total fluorescein concentration).

The kinetic experiments revealed pronounced changes in decay characteristics when going from acid to alkaline solutions, and these cases therefore will be treated separately.

ALKALINE SOLUTION. The experiments refer to solutions which are 0.01 M with respect to NaOH. The concentration of the triplet state during the decay was found to be proportional to the measured transient extinction at wavelengths of transparence of ground-state fluorescein (>530 mμ). Of the radicals appearing, the X form was shown to have an extinction coefficient of the order of 100 throughout the visible. The contributing absorption of the X form had to be considered only in the ultimate stages of the triplet reactions.

The concentration dependence of the rate of decay of the triplet was obtained from measurements of transient absorptions at 600 mμ. Decay curves at different concentrations are shown in Fig. 13. The reciprocal of the transient extinction is plotted against the time after flashing. The initial slopes are independent of the concentra-

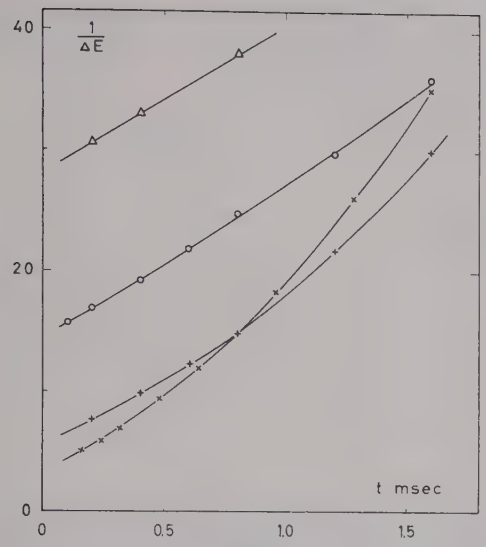


Fig. 13.

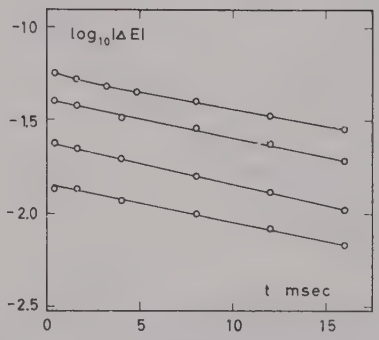


Fig. 14.

Fig. 13. The concentration dependence of the decay rate of the fluorescein triplet in alkaline solution (0.01 *M* NaOH). The reciprocal of the transient extinction at 600 mμ is plotted against the time after flashing. Fluorescein concentration, △ 0.225 μM, ○ 0.52 μM, + 1.00 μM and × 2.00 μM. Cuvette length 20 cm.

Fig. 14. The return of triplet fluorescein to the ground state after flashing, measured at the absorption peak of fluorescein, 491 mμ, in alkaline solution (0.01 *M* NaOH). The curves refer to different degrees of excitation. The extinction change (which is negative) is proportional to the concentration of intermediates. Fluorescein concentration 0.010 μM. Cuvette length 60 cm.

tion, indicating that second order reactions predominate. The second order constant in Eq. {2} is obtained as

$$k_2 + 2\,k_4 = 1.7 \times 10^9\ M^{-1}\ \text{sec}^{-1}.$$

The occurrence of the first order reaction (1) was established by measurements at very high dilution. At a fluorescein concentration of 0.024 μM, transient extinctions at 550 mμ gave a value of $k_1 = 40$ sec.$^{-1}$. The total extinction change was very low (0.01), and a more reliable determination of k_1 is reported below.

The determination of k_1 and k_2 was carried out by measurements at the wavelength of the absorption peak of ground-state fluorescein. It was assumed that the absorption of any intermediary products could be neglected compared to the very strong fluorescein absorption at this peak. The decrease in extinction on flashing is in this case proportional to the total concentration of intermediary products, according to Eq. {2.3}. Fluorescein solutions of extremely low concentrations could be studied by virtue of the strong fluorescein absorption, as illustrated in Fig. 14, which shows kinetic results for a 0.01 μM fluorescein solution at different flash energies. The second order reactions are strongly suppressed at this concentration and the curves show that a reaction of the first order governs the return of fluorescein to the ground state. The rate constant k_1 was determined to be 50 sec^{-1}.

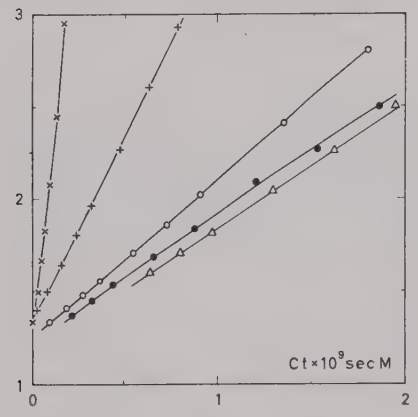


Fig. 15.

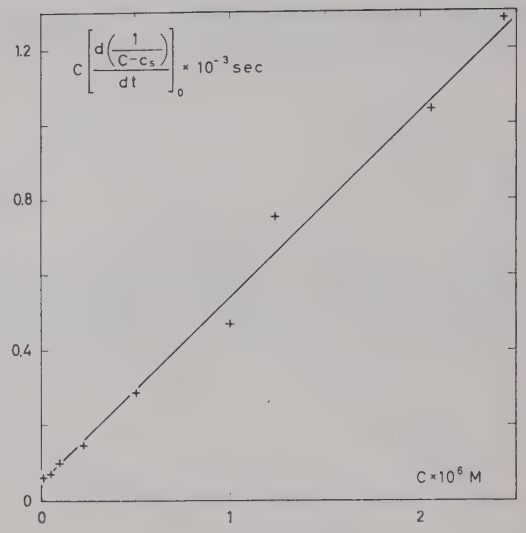


Fig. 16.

Fig. 15. Concentration dependence of the rate of recovery of ground-state fluorescein in alkaline solution (0.01 *M* NaOH). The reciprocal of the change in extinction coefficient at 491 mμ is plotted against the product of the overall fluorescein concentration and the time elapsed after flashing. Fluorescein concentration, × 0.011 μ*M*, + 0.049 μ*M*, ○ 0.225 μ*M*, ● 0.55 μ*M* and △ 2.06 μ*M*.

Fig. 16. Graphical evaluation, according to Eq. {5}, of the constants of the first and second order reactions governing the recovery of fluorescein to its ground state. The intercept gives the first order constant (50 sec^{-1}) and the slope of the line the second order constant (5.0×10^8 M^{-1} sec^{-1}).

The rate of recovery of ground-state fluorescein was studied at different concentrations. This rate may be expressed, taking reactions (1) to (7) into consideration, as

$$\frac{dc_S}{dt} = k_1 c_T + k_2 c_T^2 + (k_3 - k_5) c_T c_S + k_6 c_T c_X + k_7 c_T c_R. \qquad \{3\}$$

The initial rate of recovery is

$$\left[\frac{dc_S}{dt}\right]_0 = k_1 C + k_2 C^2. \qquad \{4\}$$

The concentration dependence of the rate of recovery is shown in Fig. 15. For easier comparison, the reciprocal of the apparent change in extinction coefficient is plotted against the product of the total concentration and time elapsed after flashing. The period of excitation (about 70 μsec) is included in this time, introducing a slight displacement of the curves along the horizontal axis when going to higher concentrations. If only second order reactions occur, the slopes should be independent of the concentration.

In the present case competing reactions apparently occur which can be resolved by the use of Eq. {4}, rearranged to suit the second order representation,

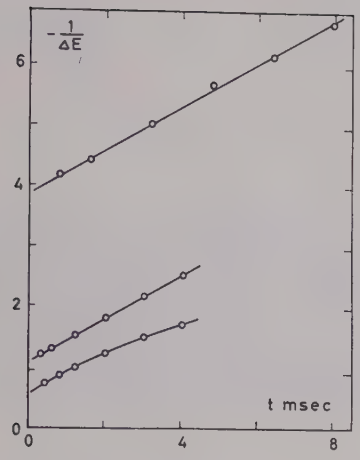


Fig. 17.

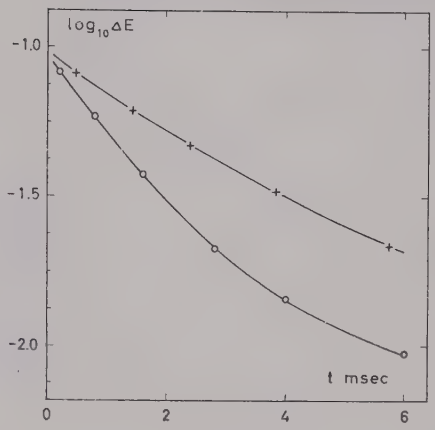


Fig. 18.

Fig. 17. Triplet quenching by unexcited fluorescein in alkaline solution (0.01 M NaOH). The reciprocal of the change in extinction at 491 mμ is plotted against the time after flashing. The curves refer to different degrees of excitation. Fluorescein concentration 1.24 μM. Cuvette length 20 cm.

Fig. 18. Triplet quenching by unexcited fluorescein in alkaline solution (0.01 M NaOH). The curve ○ refers to the transient extinction at 550 mμ measured on a 4.03 μM solution of fluorescein brought to 5 % conversion by a low energy flash. For comparison the triplet decay after complete conversion of a 0.20 μM solution to the triplet state is shown ×. Cuvette length 60 cm.

$$C\left[\frac{d\left(\frac{1}{C-c_s}\right)}{dt}\right]_0 = k_1 + k_2 C. \qquad \{5\}$$

The left hand member of Eq. {5} is plotted against the total concentration of fluorescein in Fig. 16. A straight line is obtained and the constants are determined from the slope and intercept,

$$k_1 = 50 \text{ sec}^{-1},\ k_2 = 5.0 \times 10^8\ M^{-1}\text{ sec}^{-1},\ \text{hence}\ k_4 = 6 \times 10^8\ M^{-1}\text{ sec}^{-1}.$$

Reactions (3) and (5) were studied varying the ratio between the initial concentrations of triplet and ground-state fluorescein by changing the flash light energy. Fig. 17 shows a second order plot of the changes in extinction at 491 mμ for a 1 μM fluorescein solution. The slope of the curves is independent of the initial degree of conversion. It is evident that if the reactions (3) and (5) proceed at the same rate, their occurrence will not be revealed by measurements of the initial rate of recovery of ground-state fluorescein, one S being consumed for each S recovered. The influence of reaction (1) can be neglected and one obtains, if the reactions (3) and (5) occur, the result

$$k_3 = k_5.$$

In order to get absolute values of these constants, the decay of the triplet was studied under analogous conditions. A typical result is given in Fig. 18 which shows

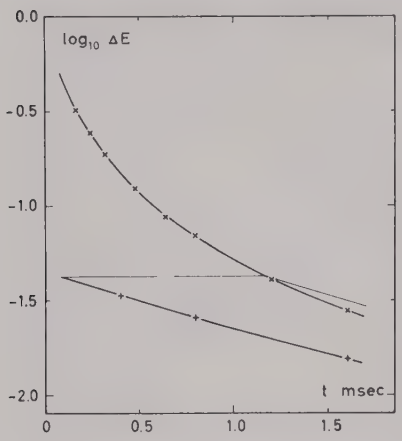


Fig. 19. Triplet quenching by fluorescein radicals in alkaline solution (0.01 M NaOH). Fluorescein concentration 4.0 μM. × triplet decay after complete conversion of fluorescein to the triplet state. + decay after a lower degree of conversion. The logarithm of the transient extinction at 600 mμ is plotted against the time after flashing. Cuvette length 20 cm. The finer lines indicate a displacement of the lower curve along the time axis.

the transient extinction at 550 mμ, for a 4 μM fluorescein solution, starting at 5 % conversion to the triplet state. For comparison, the decay after complete conversion is shown for a more dilute solution. The decay is considerably more rapid in the first case demonstrating the quenching by S molecules. An expression for the initial rate, when S is present at the beginning of the dark reaction, is obtained from a rearranged form of Eq. {1} (c_T^0 is the triplet concentration during flashing),

$$-\left[\frac{d\ln c_T}{dt}\right]_0 = k_1 + (k_3 + k_5)\,C + (k_2 + 2\,k_4 - k_3 - k_5)\,c_T^0. \qquad \{6\}$$

From the Figure is calculated

$$k_3 + k_5 = 1 \times 10^8\ M^{-1}\ \text{sec}^{-1}.$$

k_3 and k_5 were found to be equal, thus

$$k_3 = k_5 = 5 \times 10^7\ M^{-1}\ \text{sec}^{-1}.$$

The occurence of the reactions (6) and (7) which express the quenching of T by X and R, is confirmed by the kinetic results. The kinetic curves demonstrated in Fig. 13 deviate from a second order decay at an earlier stage than is accounted for by the first order reaction (1) or by the quenching reactions (3) and (5). Furthermore, the radicals were not, as will be shown, formed to the extent required by reactions (4) and (5). The quenching effect was studied at one concentration, varying the initial degree of conversion. Fig. 19 shows the results of two experiments, referring to complete and partial conversion. The logarithm of the transient extinction at 600 mμ is plotted against the time after flashing. In the absence of reactions (6) and (7), it should be possible to bring the two curves into coincidence by displacement along the time axis. This is not possible;, and from the difference between the initial rate at low conversion and the rate at the same triplet concentration when starting at high conversion, the quenching constants k_6 and k_7 may be calculated (Eq. {1}), provided the concentration of the radicals is known. It is shown in the next

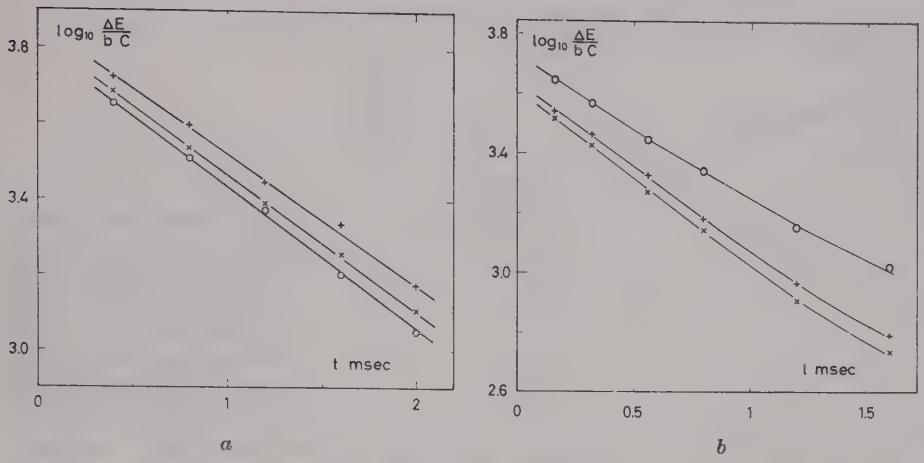


Fig. 20. pH dependence of the triplet decay in acid solutions. + pH 1.7, × pH 3.3 and ○pH 5.5. (*a*) pH dependence of the first order decay. Fluorescein concentration 0.10 μM. The logarithm of the increase in extinction coefficient at 510 mμ is plotted against the time after flashing. (*b*) pH dependence of the second order decay. Fluorescein concentration 0.25 μM. The logarithm of the increase in extinction coefficient at 540 mμ is plotted against the time after flashing.

section that each radical is formed to an extent of about 20 % and using this value one obtains

$$k_6 + k_7 = 1 \times 10^9 \; M^{-1} \; \text{sec}^{-1}.$$

Acid solution. The experiments were, if not otherwise stated, performed with solutions 0.025 M with respect to $HClO_4$. The proximity of the absorption peaks of the ground-state fluorescein and the radical form X introduces a complication in the kinetic analysis of the triplet decay in acid solution. The results indicate, however, that the reactions occurring in alkaline solution also govern the kinetics in acid solution, although with different efficiencies.

It was shown above that only one kind of absorption spectrum appeared in acid solutions. It was expected that even if the cation, neutral molecule, or mono-anion had about the same absorption spectrum, they would nevertheless differ from each other in reaction kinetics. However, no differences in the rates of triplet decay were noted in the pH range studied, 0 to 5.5. Triplet decay curves at different acidities and concentrations are shown in Fig. 20. The measurements with highly dilute solutions show that the first order decay (1) is independent of acidity. The lower extinction change observed at pH 5.5 does not correspond to a low extinction coefficient of the triplet but is due to a slight absorption of the ground-state mono-anion at the wavelength of measurement. The second order reactions are apparently also independent of the acidity, as is seen from the data obtained from studies at a higher concentration of the dye (Fig. 20*b*). Hence, from the kinetic point of view only one species is being dealt with, referred to as the acid form of the triplet.

The curves shown in Fig. 20 are indicative of a first order reaction. On the other hand, when the concentration is varied, the rate of the first order decay changes, as illustrated by experiments reported in Fig. 21. The initial rates (starting at complete conversion) are extrapolated from the logarithmic representation of the transient

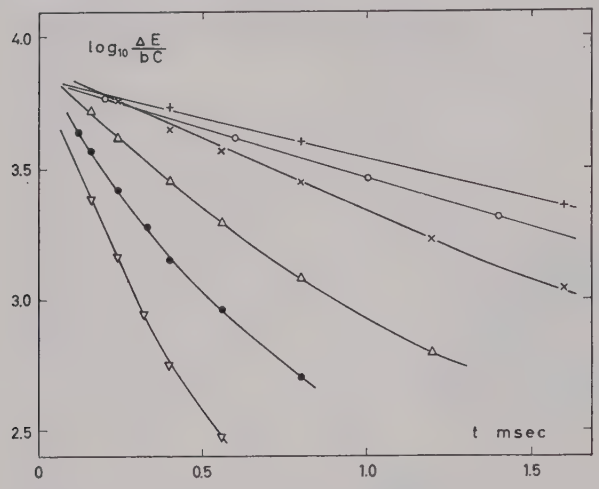


Fig. 21. Concentration dependence of the decay rate of the fluorescein triplet in acid solution (0.025 M $HClO_4$). The logarithm of the change in extinction coefficient at 510 mμ is plotted against the time after flashing. Fluorescein concentration, + 0.047 μM, ○ 0.100 μM, × 0.189 μM, △ 0.523 μM, ● 1.03 μM, and ▽ 1.99 μM.

extinction. Assuming the mechanisms (1) to (5) to be valid, the initial rate of decay is given by the expression

$$-\left[\frac{d\ln c_T}{dt}\right]_0 = k_1 + (k_2 + 2k_4)C. \qquad \{7\}$$

The constants are obtained by plotting the left hand member of Eq. {7} against the total concentration, as shown in Fig. 22, giving

$$k_1 = 600 \text{ sec}^{-1}$$
$$k_2 + 2k_4 = 3.5 \times 10^9 \ M^{-1} \text{ sec}^{-1}.$$

The mechanisms (3) and (5) were studied by varying the light energy incident on a 4 μM fluorescein solution. The initial relative rates were found to be comparatively independent of the degree of conversion, as illustrated by Fig. 23. This result implies that a T molecule reacts almost as easily with a S molecule as with another T molecule, hence the apparent first order rate. From the Figure is obtained

$$k_3 + k_5 = 2.5 \times 10^9 \ M^{-1} \text{ sec}^{-1}.$$

The quenching reactions (6) and (7) were not investigated. These reactions probably occur, but are hidden due to the high reactivity of S with T.

Reactions (4) and (5) are treated in more detail in the following section.

The radical states

Radical formation.—The appearance of two prominent absorption bands during the triplet decay indicated that triplet molecules react forming further intermediates. The absorption maxima are situated at 355 and 428 mμ in acid or weakly alkaline solution, while in strongly alkaline solution the ultraviolet band is displaced to 394 mμ. This behavior points to the existence of two secondary products, one of which is sensitive to pH. Further experiments showed that the intermediates are

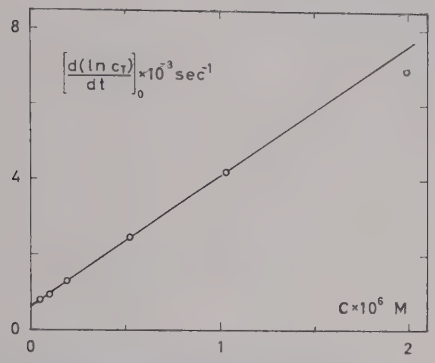


Fig. 22.

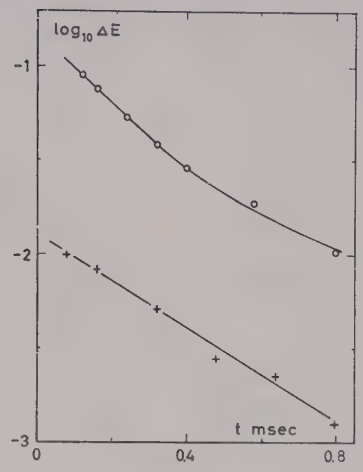


Fig. 23.

Fig. 22. Graphical determination, according to Eq, {7}, of the first and second order reactions governing the triplet decay in acid solution (0.025 M $HClO_4$). Initial rates taken from Fig. 21. The intercept gives the first order constant (600 sec^{-1}) and the slope the second order constant (3.5×10^9 M^{-1} sec^{-1}).

Fig. 23. Triplet quenching by unexcited fluorescein molecules in acid solution (0.025 M $HClO_4$). The logarithm of the transient extinction at 540 mμ is plotted against the time after flashing. Initial conversion 100 % ○, and 10 % ×. Fluorescein concentration 1.00 μM, cuvette length 20 cm.

always formed in the same ratio and in yields which are independent of the fluorescein concentration (except at very low concentrations).

As a reasonable explanation of the above-mentioned results, the occurrence of an electron transfer process was assumed, leading to the reactions

$$T + T \rightarrow S^{\cdot -} + S^{\cdot +} \tag{8}$$

or

$$T + S \rightarrow S^{\cdot -} + S^{\cdot +} \tag{9}$$

where $S^{\cdot -}$ represents a semi-reduced, and $S^{\cdot +}$ a semi-oxidized form of fluorescein. The semi-reduced form is identified as the pH-sensitive product in the Photoreduction Section. This form is denoted by R and the semi-oxidized radical by X.

The possibility of direct formation of these radicals upon photoexcitation was first considered. Fig. 24 shows the absorption spectra of an alkaline fluorescein solution during flashing and 1.6 msec after the flash. The spectrum appearing during the flash may be attributed to the triplet and does not show any sign of increased absorption near 394 or 428 mμ. It was concluded that the radicals are not formed as primary photoproducts. The spectrum obtained after the flash shows the replacement of the triplet absorption by the radical bands.

The triplet-singlet reaction (9) should be of importance in low-intensity photolysis and was therefore subjected to a more detailed study. An alkaline fluorescein solution was exposed to different light energies and the initial triplet concentration compared with the concentration of radicals formed, Fig. 25. The triplet concentration during flashing was obtained from the extinction changes at 550 mμ. The extinc-

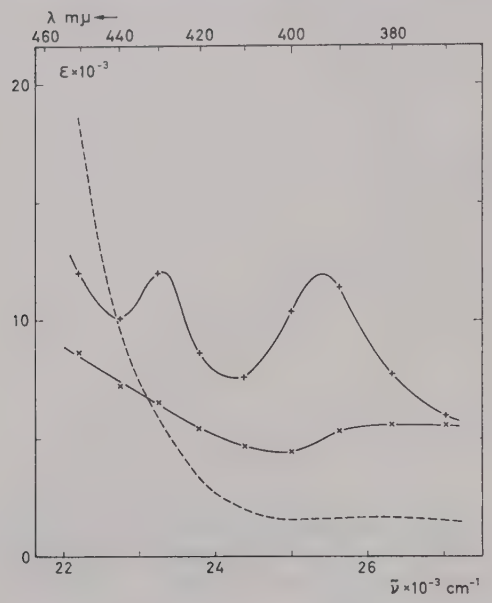


Fig. 24. The absorption spectrum of a solution of 0.5 μM fluorescein in 0.01 M NaOH; – – – before flashing; × during flashing, + 1.6 msec after the flash.

tion change at 394 mμ due to semi-reduced radicals, measured at the maxima of the corresponding kinetic curves, is plotted against the initial degree of conversion to the triplet state. (The corresponding curves in Figs. *a* and *b* refer to equal light energies.) The radical yield does not depend upon the degree of conversion and, consequently, mechanisms (8) and (9) are both occurring. It is also seen that these reactions compete with the corresponding quenching reactions (2) and (3) respectively with about equal efficiency. The deviation from linearity of the curve shown in Fig. 25*c* was not considered significant.

An analogous experiment was carried out in acid solution. Kinetic results for a solution of fluorescein in 0.025 M $HClO_4$ are shown in Fig. 26. The radical concentration was measured at 355 mμ and the triplet concentration at 510 mμ. It is apparent from the Figure that the conclusions drawn in the case of alkaline solutions are applicable also to acid solutions.

The kinetics of the radical reactions were studied by recording the transient extinction changes at the absorption peaks of the two radicals. It was for this purpose necessary to determine the absolute extinction coefficients of the two radical forms at these maxima. The radical concentration was estimated from measurements of the decay of triplet and the simultaneous recovery of ground-state fluorescein. In Fig. 27 results for a 0.5 μM fluorescein solution in 0.01 M NaOH are reported. The change in extinction coefficient at 540 mμ gives a measure of the concentration of triplet, and at 491 mμ a measure of the total concentration of the intermediate products. The extinction changes at 428 and 394 mμ indicate that the radical concentration reaches its maximum about 2 msec after the flash. At this moment 33 % of the fluorescein is in the ground state and 32 % in the triplet state. This leaves about 18 % for each of the radical forms. This figure is obtained neglecting the subsequent reactions of the radicals and represents a lower limit for the formation of each radical species. From the values of the rate constants for the reactions (4) and (5) a higher percentage of

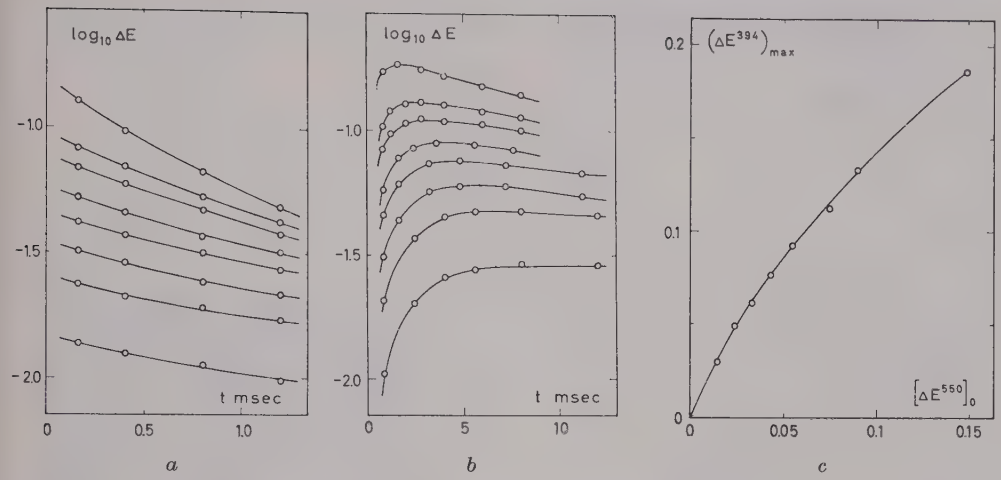


Fig. 25. Formation of semi-reduced fluorescein radicals in alkaline solution as a function of the initial degree of conversion of fluorescein to the triplet state. Fluorescein concentration 1.02 μM (in 0.01 M NaOH). Corresponding curves in Figs. (*a*) and (*b*) refer to equal light energies. Cuvette length 20 cm.

(*a*) The logarithm of the transient extinction at 550 mμ plotted against the time after flashing. (*b*) The logarithm of the extinction change at 394 mμ plotted against the time after flashing. (*c*) Maximum extinction change at 394 mμ, plotted against the change in extinction at 550 mμ during flashing.

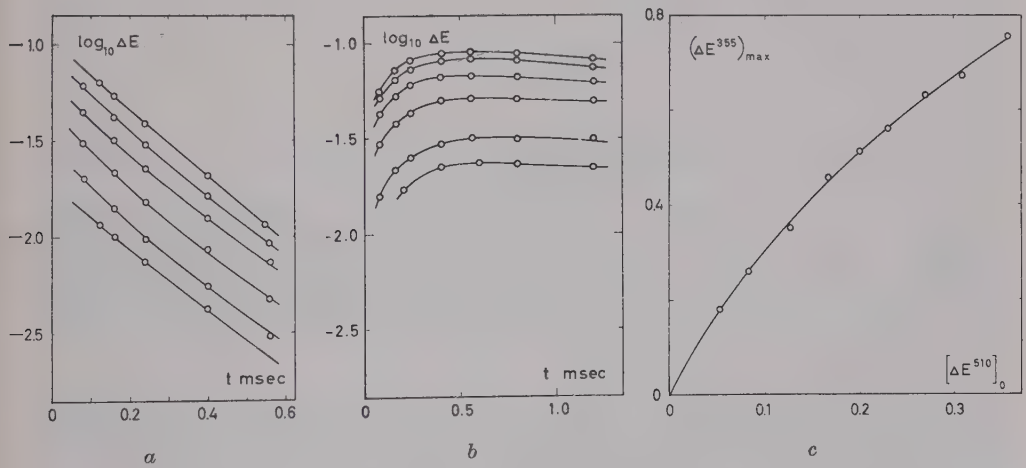


Fig. 26. Formation of semi-reduced fluorescein radicals in acid solution as a function of the initial degree of conversion of fluorescein to the triplet state. Fluorescein concentration 1.05 μM (in 0.025 M $HClO_4$). Corresponding curves in Figs. (*a*) and (*b*) refer to equal light energies. Cuvette length 20 cm.

(*a*) The logarithm of the transient extinction at 510 mμ, plotted against the time after flashing. (*b*) The logarithm of the extinction change at 355 mμ plotted against the time after flashing. (*c*) Maximum extinction change at 355 mμ, plotted against the change in extinction at 510 mμ during flashing. This curve refers to a 2.08 μM fluorescein solution, cuvette length 60 cm.

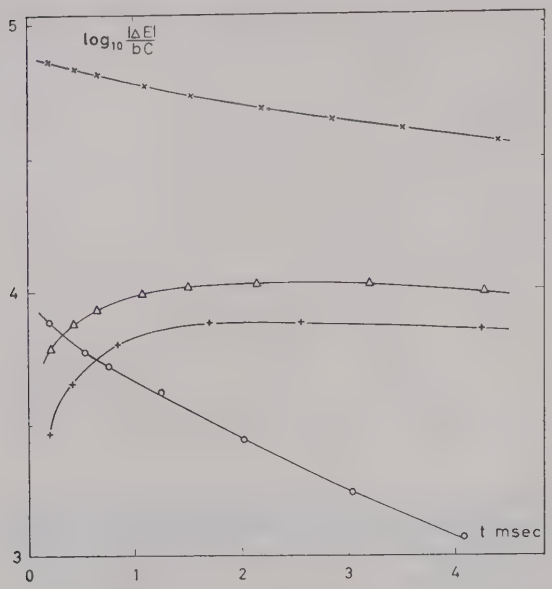


Fig. 27. Extinction changes on flashing a 0.50 μM alkaline solution of fluorescein (0.01 M NaOH), measured at wavelengths characteristic for the semi-reduced state (394 mμ) △, the semi-oxidized state (428 mμ) +, the ground state (491 mμ) ×, and the triplet state (540 mμ) ○ of fluorescein. The logarithm of the change in extinction coefficient is plotted against the time after flashing. The change is negative at 491 mμ.

radicals is expected. The quenching effect of the radicals on the triplet state, expressed by reactions (6) and (7), explains the apparent disagreement.

The determination of the relative contribution of the two radical forms to the absorption changes at 394 and 428 mμ required supplementary information which was obtained from measurements at a lower pH. The pH dependence of the R form is treated in detail in the last paragraph of this section. These experiments showed that R exists in an acid form with a peak at 355 mμ, and in a base form with a peak at 394 mμ. The pK_a value was estimated to be 9.5. The rate of radical formation also was shown to be independent of pH at pH higher than 8 (i.e., in the pH range where only the base form of the triplet appears).

Assuming that the absorption of the *acid* form of R is negligible at 428 mμ, a comparison between the extinction changes at 428 mμ, studied in the pH ranges of existence of the acid and base forms of R respectively, should give the extinction coefficient of the *base* form of R. Such measurements, carried out at pH 8.1 and 12, are shown in Fig. 28*a*. The slight increase in extinction at pH 12 is due to the absorption of the base form of the radical and one obtains $\varepsilon_R = 5 \times 10^3$ at 428 mμ.

The contribution of X to the absorption peak at 394 mμ was also determined by comparing the results of measurements at pH 8.1 and 12, Fig. 28*b*. It was assumed that at 394 mμ the absorption of the acid form of R could be neglected. After a correction for the 5 % of R appearing in the base form at pH 8.1, one obtains the extinction coefficient of X, $\varepsilon_X = 8 \times 10^3$ at 394 mμ.

It was also necessary to take into account the absorption of triplet molecules at the two absorption peaks. From Fig. 24 values of $(\varepsilon_T - \varepsilon_S)$ were obtained, amounting to 1×10^3 at 428 mμ and to 3×10^3 at 394 mμ.

The extinction change when T, R and X are present, is given by the expression

$$\Delta E = b[c_T(\varepsilon_T - \varepsilon_S) + c_R(\varepsilon_R - \varepsilon_S) + c_X(\varepsilon_X - \varepsilon_S)].$$

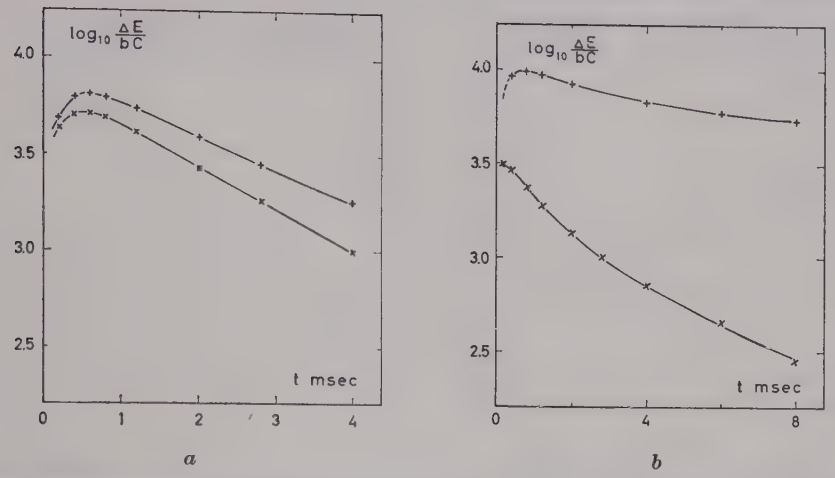


Fig. 28. Comparison between extinction changes at pH 8.1 and 12. Fluorescein concentration 2.0 μM, cuvette length 20 cm.
(*a*) The logarithm of the change in extinction coefficient at 428 mμ is plotted against the time after flashing, × pH 8.1, + pH 12. (*b*) The logarithm of the change in extinction coefficient at 394 mμ is plotted against the time after flashing, × pH 8.1, + pH 12.

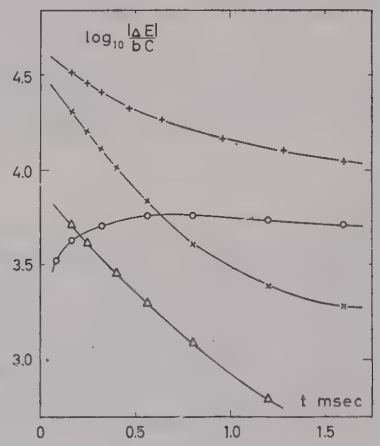


Fig. 29. Extinction changes on flashing a 0.50 μM acid solution of fluorescein (0.025 M $HClO_4$), measured at wavelengths characteristic for the semi-reduced state (355 mμ) ○, the semi-oxidized state (428 mμ) ×, the ground state (437 mμ) ×, and the triplet state (510 mμ) △ of fluorescein. The logarithm of the change in extinction coefficient is plotted against the time after flashing. The change is negative at 428 and 437 mμ.

The extinction coefficients of the two radical species can be calculated from the above formula. In Fig. 27 extinction changes at 394 and 428 mμ are shown. From the changes observed 2 msec after flashing one obtains $\varepsilon_R = 5 \times 10^4$ at 394 mμ and $\varepsilon_X = 5 \times 10^4$ at 428 mμ. These figures agree well with the values obtained from the photoreduction and photo-oxidation experiments.

The kinetic data referring to acid solutions cannot be treated in an analogous manner because the peaks of the fluorescein in the ground state and in the semi-oxidized state lie too close together. Instead, the approximate value of 3×10^4 for the extinction coefficient of R at the ultraviolet peak (355 mμ), obtained from photo-

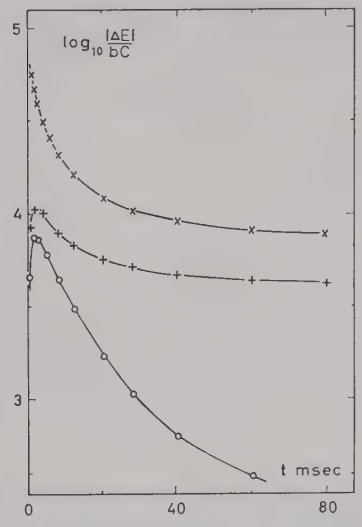


Fig. 30.

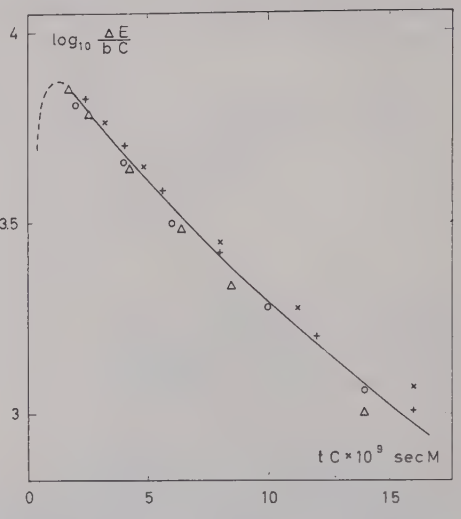


Fig. 31.

Fig. 30. The disappearance of the radical states of fluorescein in alkaline solution (0.01 *M* NaOH). The logarithm of the change in extinction coefficient is plotted against the time after flashing, at wavelengths 394 mμ +, 428 mμ ○, and 491 mμ ×. Fluorescein concentration 0.50 μM. The change is negative at 491 mμ.

Fig. 31. Concentration dependence of the rate of disappearance of the radical states of fluorescein in alkaline solution (0.01 *M* NaOH). The logarithm of the change in extinction coefficient at 428 mμ is plotted against the product of the overall fluorescein concentration and the time after flashing. Fluorescein concentration △ 0.53 μM, ○ 1.04 μM, + 2.04 μM, × 4.08 μM.

reduction experiments, was used. The formation of radicals in acid solution is demonstrated by an experiment carried out with a 0.5 μM solution of fluorescein in 0.025 *M* $HClO_4$. Extinction changes on flashing were recorded at 355, 428, 437 and 510 mμ, and the results are shown in Fig. 29. At 428 mμ, the increase in absorption due to the appearance of the *X* form is exceeded by the decrease in absorption due to the decrease in concentration of the ground-state fluorescein, which absorbs strongly at this wavelength. The maximum concentration of *R*, and consequently also of *X*, is calculated to be about 20 % of the initial fluorescein concentration. This gives an extinction coefficient of 7×10^4 for *X* at 428 mμ, which agrees comparatively well with the value obtained for alkaline solutions.

Radical reactions.—With the acquired knowledge about the extinction coefficients of the radicals, it is possible to study the kinetics of the radical reactions in more detail. The disappearance of the radicals in alkaline solution is illustrated by the experiment reported in Fig. 30. The Figure shows measurements of extinction changes at 394, 428 and 491 mμ. It is immediately apparent from the curves that the semi-reduced and semi-oxidized fluorescein molecules do not disappear at the same rate. The formation of a third product with a strong absorption maximum at the same wavelength as the semi-reduced form cannot be excluded but seems unlikely and is not considered in the following discussion. Initially *X* and *R* show similar decay

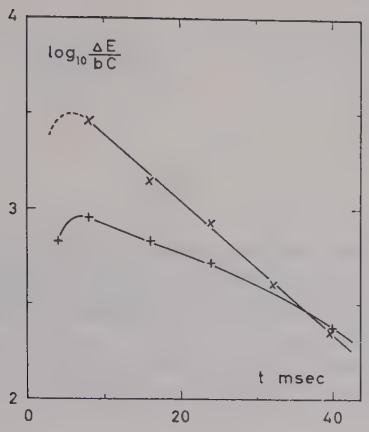


Fig. 32.

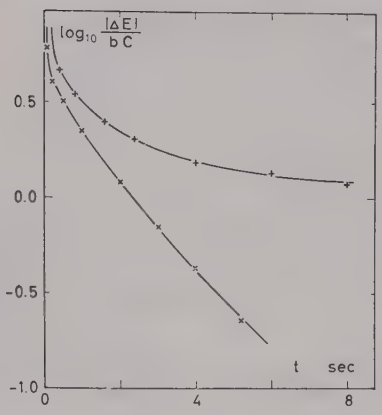


Fig. 33.

Fig. 32. Demonstration of the second order disappearance of the semi-oxidized form of fluorescein. The logarithm of the extinction change at 428 mμ is plotted against the time after flashing; × high conversion to the triplet state, + low conversion. Fluorescein concentration 0.49 μM (0.01 M NaOH), cuvette length 60 cm.

Fig. 33. The last stage of the flash-initiated reactions occurring in alkaline fluorescein solution (0.01 M NaOH). The extinction change is plotted against the time after flashing, at the wavelengths 394 mμ ×, and 491 mμ +. Fluorescein concentration 0.50 μM, cuvette length 60 cm. 3.0 should be added to the figures on the vertical axis.

characteristics, but as the reaction proceeds, X is depleted more rapidly than R. Possible mechanisms explaining this behavior would be

$$X + R \rightarrow S + S \tag{10}$$

and

$$X + X \rightarrow X_2. \tag{11}$$

The occurrence of these reactions is supported by the extinction changes observed at 491 mμ (Fig. 30).

Experiments at different fluorescein concentrations confirmed the existence of only second order reactions causing the removal of the radicals. Fig. 31 shows the changes in extinction coefficient at 428 mμ, at fluorescein concentrations of 0.5 to 4 μM (in 0.01 M NaOH), plotted against the product of total concentration and time after flashing. The different sets of points can be represented by the same curve, as expected if only second order reactions are involved.

The possibility of an association reaction

$$X + S \rightarrow X, S \tag{12}$$

could be disregarded, as shown by the following experiment. A 0.5 μM solution of fluorescein in 0.01 M NaOH was exposed to different light energies and the extinction changes at 428 mμ measured. X appears in low concentration and the concentration of ground-state fluorescein may therefore be considered constant during the disappearance of X. Consequently, the association reaction should be of the first order. The results shown in Fig. 32 indicate that a second order reaction is responsible for the removal of X, as required if the mechanisms (10) or (11) occur.

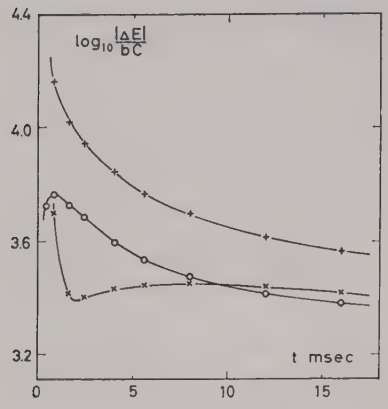


Fig. 34. The disappearance of the radical states of fluorescein in acid solution (0.025 *M* $HClO_4$). The logarithm of the change in extinction coefficient is plotted against the time after flashing, at the wavelengths 355 mμ ○, 428 mμ ×, and 437 mμ +. At 428 and 437 mμ the extinction is decreased on illumination. Fluorescein concentration 0.50 μM.

Determination of the rate constants of reactions (10) and (11) requires knowledge of the absorption spectrum of the dimer form of *X* or other products that may appear. The decomposition of fluorescein on flashing (4 %) obscured the results at this stage of the reaction and the spectrum of X_2 could not be determined. It was, therefore, not attempted to evaluate the rate constants. A rough estimate shows that the order of magnitude of k_{10} and k_{11} is 10^9 M^{-1} sec^{-1}.

When the semi-oxidized form had disappeared, the decay of the semi-reduced form became very slow. The *S* form was observed to reappear at approximately the same slow rate. Fig. 33 illustrates the final disappearance of *R*, measured at 394 mμ and the appearance of *S*, measured at 491 mμ. The only species in the reaction mixture that could re-oxidize *R* to the original fluorescein seems to be the dimer form X_2. The reaction mechanism for the slow step could possibly be

$$2R + X_2 \rightarrow 4S. \qquad (13)$$

In acid solutions reactions analogous to those in alkaline solutions apparently occurred, the *X* form being consumed more rapidly than the *R* form. This is illustrated by Fig. 34 which shows the extinction changes for a 0.5 μM fluorescein solution in 0.025 *M* $HClO_4$, at wavelengths 355, 428 and 347 mμ, corresponding to the absorption peaks of *R*, *X* and *S* respectively.

At 428 mμ the disappearance of the *X* absorption band is balanced by the reappearance of the *S* band (at 437 mμ), and only a slight extinction change is observed during the decay of *X*. The decay of *X* is illustrated by the disappearance of the intermediary products studied at 437 mμ.

The final disappearance of the *R* form was also slow in acid solution. It was believed that the reaction (13) was responsible for the recovery of the fluorescein but no detailed measurements were made because of the interference of the permanent photoproducts.

pH dependence.—The radical spectra were studied in the pH range 0–13. No significant change in position or absorption intensity of the peak attributed to the semi-oxidized form could be detected over this pH interval. The absorption peak of the semi-reduced form, on the other hand, changed from 355 mμ in acid or weakly alkaline solution to 394 mμ in strongly alkaline solution. This change probably is due to dissociation of an acid form of the semi-reduced fluorescein to the conjugate base.

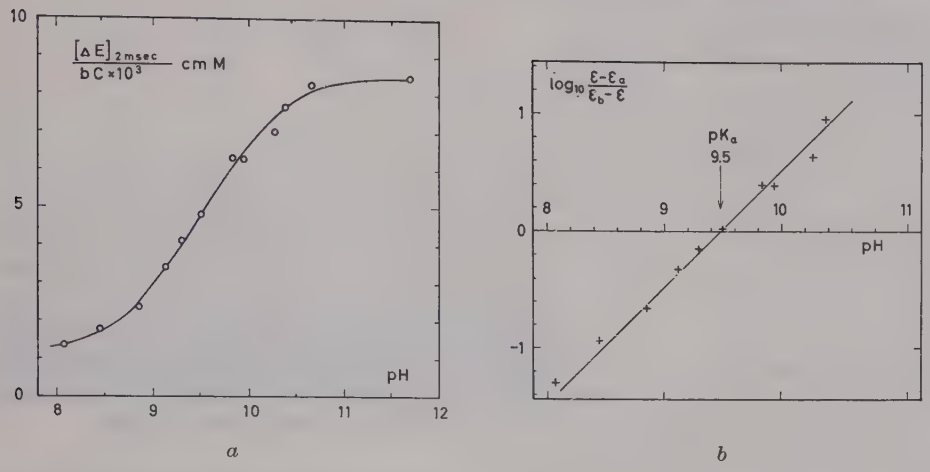


Fig. 35. The dissociation equilibrium of the semi-reduced form of fluorescein. Fluorescein concentration 2.0 μM, cuvette length 60 cm.
(*a*) The change in extinction coefficient at 394 mμ, observed 2 msec after flashing, as a function of pH. (*b*) Graphical determination of the pK_a value of the semi-reduced form, according to formula {3.1}. A straight line with slope 1 is fitted to the points. pK_a = 9.5.

The dissociation constant of the acid was obtained from a series of experiments conducted between pH 8 and 12, demonstrated in Fig. 35. Extinction changes on flashing were recorded at 394 mμ, corresponding to the peak of the base form of *R*. In Fig. 28*b* two kinetic curves are shown. The apparent change in extinction coefficient 2 msec after the flash is plotted against pH, and the curve obtained indicates a pK_a value between 9 and 10. A more exact value of pK_a was obtained by the use of Eq. {3.1}. $\text{Log}_{10}(\varepsilon-\varepsilon_a)/(\varepsilon_b-\varepsilon)$ is plotted against pH; the intercept of the resulting curve with the pH axis gives pK_a = 9.5.

Irreversible reactions

Solutions of fluorescein in water are very stable when kept in the dark. Irradiation by visible light of normal intensities has no effect upon oxygen-free solutions, even after prolonged exposure, while photobleaching occurs in the presence of oxygen. However, at the high light intensities achieved in the flash photolysis experiments, the dye is slighly decomposed by visible light even in the absence of oxygen. The fluorescein absorption peak in the visible is depressed and a region of absorption appears on the low-frequency side of the peak. The colour becomes reddish in alkaline solution and orange in acid solution. The colour darkening is accompanied by the disappearance of fluorescence. The absorption spectrum of a deaerated solution of 10 μM fluorescein in 0.01 *M* NaOH, exposed to 10 flashes, each with an electrical energy of 6000 J, is shown in Fig. 36. In the presence of oxygen photodecomposition also occurs upon flashing but in decreased yields. The absorption spectrum of an air-saturated fluorescein solution also is traced in the Figure. It is apparent from the study of the absorption spectra that the nature of the photoproducts is independent of the presence of oxygen in the solution. In acid solution a similar behavior is

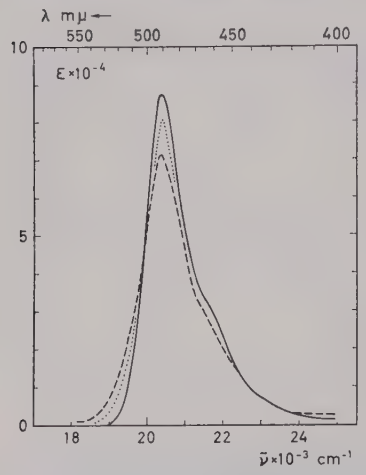


Fig. 36. The permanent change of the absorption spectrum of fluorescein in alkaline solution (0.01 *M* NaOH). —— before flashing; --- after 10 flashes (6000 J), deaerated solution; · · · after 10 flashes, air-saturated solution. Fluorescein concentration 10 μM.

encountered. Spectra of the pure photoproducts in acid and alkaline solutions are shown in Fig. 42.

The anomalous decomposition of fluorescein in oxygen-free solutions at high light intensities was investigated in a series of experiments. Due to the high concentration of triplet molecules during flashing, encounters between triplet molecules are possible, and an irreversible reaction might occur,

$$T + T \rightarrow \text{products}. \tag{14}$$

It has been shown that radical forms (X and R) of fluorescein appear during photolysis of the dye as a result of encounters between triplet molecules or between triplet and singlet molecules. Photoexcitation of these radicals, which absorb visible light, might lead to formation of permanent products,

$$\begin{aligned} X + h\nu &\rightarrow \text{products} \\ R + h\nu &\rightarrow \text{products}. \end{aligned} \tag{15}$$

Twofold excitation of fluorescein via the first excited singlet state may be excluded in view of the short lifetime of this state. Excitation of the long-lived triplet molecules has to be considered, however, and decomposition of the excited triplet (T^*) might occur,

$$T + h\nu \rightarrow T^* \rightarrow \text{products}. \tag{16}$$

It was observed that the yield of stable products is independent of the concentration. This finding rules out process (15) as in this case the concentration of radicals during the flash should increase with the concentration of fluorescein, enhancing the yield. If reaction (14) is responsible for the permanent change, the yield of photoproducts should be independent of the light energy once complete conversion to the triplet state is reached. On the other hand, in the case of the validity of reaction (16), the yield should increase with increasing light energy. Fig. 37 shows permanent

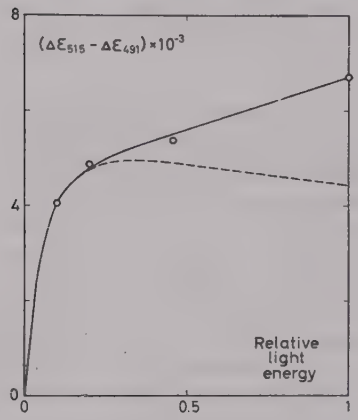


Fig. 37. Irreversible change of fluorescein in alkaline solution (0.01 M NaOH) as a function of the incident light energy. The sum of the permanent extinction decrease at 491 mμ and the increase at 515 mμ after one flash is plotted against the light energy ○. Fluorescein concentration 0.50 μM. The electrical energy was 12,000 J, and the light energy was varied by means of screens. For comparison, the conversion to triplet state as a function of the light energy (see Fig. 10) is also shown (in arbitrary units) ——.

absorption changes produced by flashes of different light energies. The electrical energy was 12,000 J, and the light intensity was varied by means of screens. The energy dependence of the degree of conversion to the triplet state on flashing (Fig. 10) is shown for comparison. It is seen that the yield of permanent products does not reach a constant value, although complete conversion to the triplet state is already achieved at about half of the maximum energy. The shape of the decomposition curve does not permit a definite conclusion concerning the mechanism of the reaction. The curve representing the degree of conversion to triplet indicates, however, that triplet molecules are consumed during the flash, supporting the occurrence of reaction (16).

If excited triplet molecules are responsible for the formation of photoproducts, a reasonable primary step would be the photoejection of an electron,

$$T + h\nu \rightarrow T^* \rightarrow S^{\cdot +} + e^- \qquad (17)$$

where $S^{\cdot +}$ represents the semi-oxidized form of fluorescein (X), the existence of which was established in connection with the treatment of the secondary reactions of the triplet of fluorescein. The photo-oxidation experiments, reported on p. 122, indicate that when oxygen is present the first product observed after flashing is the semi-oxidized form of fluorescein. If the semi-oxidized radical is the primary photoproduct also in deaerated solution, the observation that the same permanent products are obtained in deaerated as well as in aerated solutions would be explained.

The further reactions of the semi-oxidized fluorescein radicals, ending in the formation of permanent products, are dealt with in connection with the photo-oxidation experiments.

The fraction of the fluorescein decomposed per flash was determined by comparing the amounts of triplet formed by two successive flashes. At an electrical energy of 6000 J, a decomposition of about 4 % occurs in the fluorescein concentration range 0.1 to 10 μM, irrespective of the acidity. Measurements of the decrease in absorption intensity of the peak at 491 mμ, for alkaline solutions, also give a decomposition of 4 % if the decomposition products are assumed to have the absorption spectrum shown in Fig. 42.

Photoreduction of fluorescein

The results from the detailed analysis of the reversible reactions of photoactivated fluorescein formed the basis for the study of the reaction mechanisms governing photoinduced reduction of fluorescein in the presence of reducing agents. If the quantum yield for photobleaching is low the reactions observed on flashing fluorescein in pure water solution will not be appreciably influenced by the introduction of a reductant. Ethanol, for instance, was found not to affect the kinetics, even when present in high concentration. In order to obtain a positive effect comparatively strong reducing agents are required. Allyl-thiourea proved suitable in this respect and its effect on the photoreactions of fluorescein was studied.

A reversible reduction using *p*-phenylenediamine as reductant was also investigated. The latter compound is known as a strong inhibitor of photoreactions, and no net reduction is observed in its presence. The inhibitory effect is, however, due to a reversible photoreduction of the dye and is therefore treated in this section.

Irreversible reduction

Allyl-thiourea does not absorb in the visible and its action in the photoreduction of fluorescein induced by visible light is explained as an attack on the photoactivated species of the dye. The relative importance of the triplet and the radical states of fluorescein in the reduction process was determined by comparing the kinetics after flashing fluorescein in the presence and absence of reductant. Extinction changes on flashing were measured at three wavelengths, 394, 428 and 600 mμ, corresponding to the characteristic bands of the semi-reduced and semi-oxidized radicals and the triplet, respectively. The results are shown in Fig. 38. From the difference between the rates of disappearance of the triplet, in the presence and absence of reducing agent, the rate constant for the reaction between the triplet and the reductant (ATU),

$$T + ATU \begin{matrix} \nearrow R + (ATU)_{ox} \\ \searrow S + ATU \end{matrix} \qquad (k_{18}) \qquad (18)$$

is determined, $k_{18} = 2 \times 10^5\ M^{-1}\ \text{sec}^{-1}$. $(ATU)_{ox}$ refers to the primary oxidation product of the reductant. The reductant may, however, in addition to the reaction with the triplet also react with the semi-oxidized form of fluorescein formed by the electron transfer reactions (8) and (9), which compete with reaction (18). The extinction change observed at 428 mμ shows that the semi-oxidized form, being a strong oxidizing agent, reacts very rapidly with the reductant, probably by the reaction

$$X + ATU \rightarrow S + (ATU)_{ox}. \qquad (19)$$

The semi-reduced form of fluorescein is left behind, as is apparent from the runs at 394 mμ. This form probably disappears by disproportionation under formation of the leuco-dye, fluorescin. The absorption of the oxidation products of the allyl-thiourea hindered the study of the disappearance of the semi-reduced form.

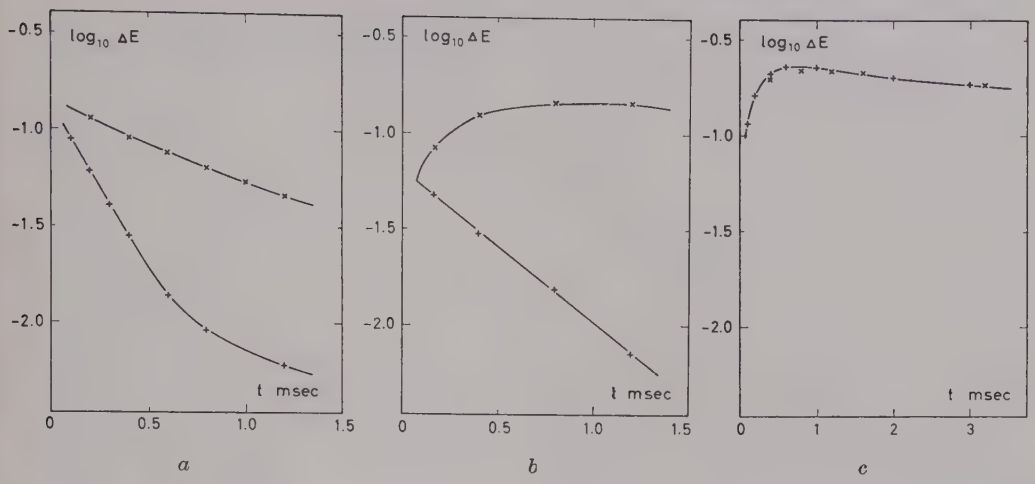


Fig. 38. Photoreduction of fluorescein with allyl-thiourea as reductant, in alkaline solution (0.01 *M* NaOH). Fluorescein concentration 1.0 μM, allyl-thiourea 0.010 *M*. Cuvette length 20 cm. The logarithms of the extinction changes are plotted against the time after flashing +. For comparison, corresponding experiments performed in the absence of reductant are also shown ×.
(*a*) Extinction change at 600 mμ, corresponding to the fluorescein triplet. (*b*) Extinction change at 428 mμ, corresponding to the semi-oxidized form of fluorescein. (*c*) Extinction change at 394 mμ, corresponding to the semi-reduced form of fluorescein.

Reversible reduction

It is known that certain substances which function as inhibitors, retarders, or accelerators of photosensitized reactions are very efficient even when present at very low concentrations. Such substances are believed to influence excited sensitizer molecules by a reduction-oxidation process.

The action of the inhibitor *p*-phenylenediamine on the intermediary photoproducts of fluorescein was studied. In deaerated solutions the fluorescein triplet formed on flashing was rapidly converted to semi-reduced radicals in the presence of *p*-phenylenediamine. The reduction was found to be reversible (0.2 % permanent change at a flash energy of 6000 J). In acid solution a strong peak at 355 mμ appeared, which in alkaline solution was displaced to 394 mμ, corresponding exactly to the positions of the peaks of the *R* form reported in the preceding section. A peak at about 320 mμ and a region of low absorption between 400 and 500 mμ also appeared, and were attributed to the semi-quinonoid form of *p*-phenylenediamine. Fig. 39 shows the absorption spectra of the reaction mixtures obtained on flashing acid and alkaline fluorescein solutions containing excess *p*-phenylenediamine. It was established in separate experiments that at this electrical energy complete conversion to the semi-reduced state is achieved, and absolute extinction coefficients were consequently obtained.

If the absorption of the semi-oxidized form of *p*-phenylenediamine is neglected at the wavelengths of the strong absorption peaks of the acid and base forms of *R*, the extinction coefficients for the acid form of *R* will be 3×10^4 at 355 mμ, and for the base form 4×10^4 at 394 mμ.

p-Phenylenediamine does not absorb in the visible and reacts exclusively by attack-

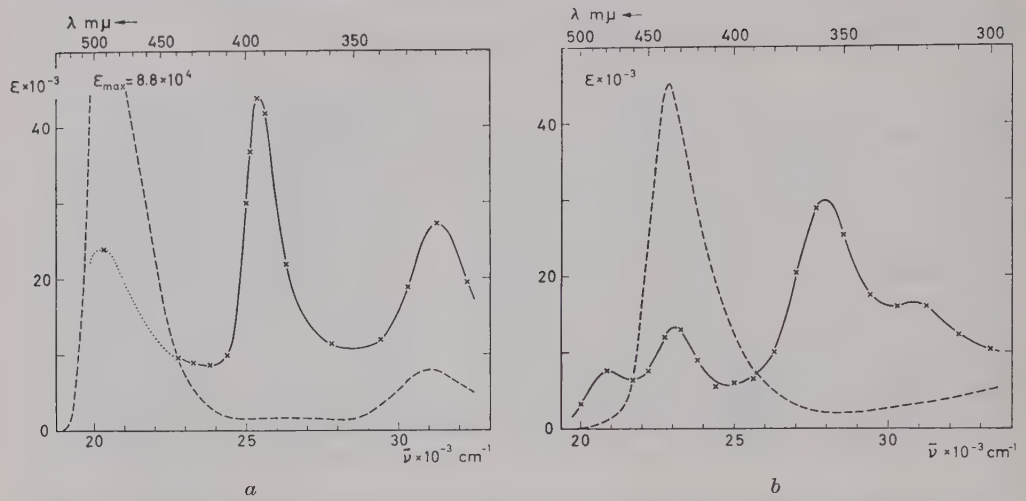


Fig. 39. Reversible photoreduction of fluorescein with *p*-phenylenediamine as reductant. The absorption spectrum appearing during flashing a 1.0 μM fluroescein solution, 10 μM with respect to the reductant, is shown ×. For comparison, the spectrum before flashing is included ----. (*a*) The spectrum of an alkaline solution, 0.01 *M* NaOH. (*b*) The spectrum of an acid solution, 0.025 *M* $HClO_4$.

ing activated species of fluorescein. At low concentrations of the inhibitor the reversible photoreduction is assumed to proceed according to the following reaction scheme,

$$T + PPA \rightarrow R + (PPA)_{ox} \qquad (k_{20}) \qquad (20)$$

$$R + (PPA)_{ox} \rightarrow S + PPA \qquad (k_{21}) \qquad (21)$$

where PPA represents *p*-phenylenediamine and $(PPA)_{ox}$ the semi-oxidized form of the inhibitor.

During the preparation of the solutions a slight oxidation of the diamine could not be avoided, and prior to the detailed investigation of the kinetics a photoreduction experiment with a partially oxidized solution of *p*-phenylenediamine was carried out. It was established that the kinetics were not influenced by the coloured oxidation products of the diamine.

Reaction (20) was studied by following the decay of the triplet and the appearance of the R band in the presence and absence of reducing agent. Fig. 40 shows results obtained in alkaline solution (0.01 *M* NaOH). The triplet decay was measured at 600 mμ and the appearance of R at 394 mμ. From the difference in initial rates of decay of the triplet, with and without reducing agent, the rate constant k_{20} was found to be approximately 5×10^9 M^{-1} sec^{-1}. It is apparent from the Figure that when *p*-phenylenediamine is present, the R peak appears and disappears more rapidly than in the absence of the inhibitor. Triplet quenching, as expressed by reactions (2) and (3), compete with the reduction and complete conversion to the R form is not reached.

The primary stage in the photoreduction is probably expressed by reaction (20).

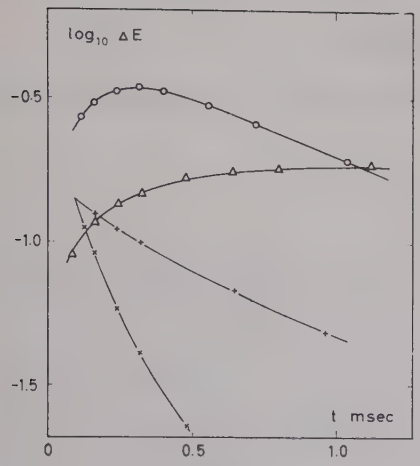


Fig. 40.

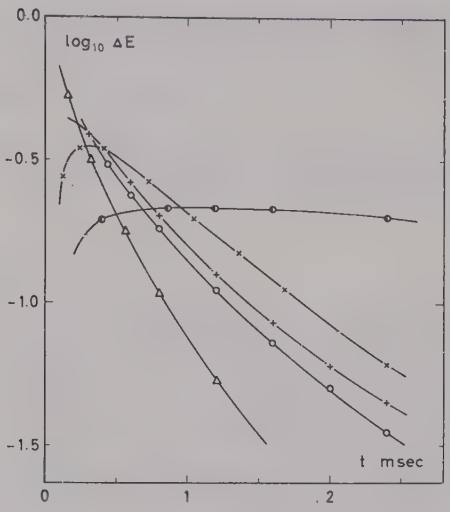


Fig. 41.

Fig. 40. Reversible photoreduction of fluorescein in alkaline solution (0.01 M NaOH) with p-phenylenediamine as reductant. Fluorescein concentration 1.05 μM, concentration of reductant 1.0 μM, The logarithms of the extinction changes at 600 mμ × and 394 mμ ○ are plotted against the time after flashing. For comparison, the corresponding curves observed in the absence of reductant are also shown, 600 mμ +, and 394 mμ △. Cuvette length 20 cm.

Fig. 41. The disappearance of the semi-reduced form of fluorescein in alkaline solution (0.01 M NaOH) as a function of the concentration of p-phenylenediamine. The logarithm of the change in extinction coefficient at 394 mμ is plotted against the product of the concentration of p-phenylenediamine and the time after flashing. Fluorescein concentration 1.0 μM, concentration of p-phenylenediamine 1.0 μM ×, 2.0 μM +, 10 μM ○, 100 μM △ and zero ◐. Cuvette length 20 cm.

The peaks attributed to R, however, may be due to a dimer form of R or to a complex of R with the parent molecule, as expressed by the reactions

$$R + R \rightarrow R_2 \tag{22}$$

$$R + S \rightarrow R, S. \tag{23}$$

Experiments carried out with very dilute fluorescein solutions definitely showed that when p-phenylenediamine was present in excess, the R band appeared at its full intensity during the flash, before the diffusion limited reactions (22) and (23) could occur. R consequently represents the monomeric form of the semi-reduced fluorescein.

The radical recombination (21) was studied at different concentrations of the solutes. Fig. 41 shows extinction curves at 394 mμ for 0.01 M NaOH solutions, 1 μM with respect to fluorescein and 0 to 100 μM with respect to p-phenylenediamine. The results show that the disappearance of R does not depend upon the concentration of the reducing agent, once this is present in excess. This concentration independence would be expected if only reaction (21) is responsible for the withdrawal of R.

A more detailed study revealed that side reactions of minor importance should also be considered, particularly the reaction

$$(PPA)_{ox} + PPA \rightarrow \text{products}.$$

In this communication which deals with the photochemistry of fluorescein, these secondary reactions will not be treated.

Photo-oxidation of fluorescein

In the presence of oxidizing agents, fluorescein changes irreversibly upon flashing, and a coloured, non-fluorescent product is formed. It was shown that the nature of the product is independent of the oxidant added (oxygen, ferric ions and peroxydisulphate ions were used) and of the acidity of the solution. Changes in acidity affect the colour of the photoproduct, yet this was explained by the occurrence of different ionic forms of the same product. Absorption spectra of the permanent product are shown in Fig. 42. The spectrum in acid solution was measured after exposing a 10 μM solution of fluorescein, 10^{-3} M with respect to ferric ions, to 10 flashes (6000 J each). It was shown that after this number of flashes no more decomposition occurred and that the fluorescence had disappeared. The spectrum of the degraded solution, hence, probably refers to the pure photoproduct. The above solution was made alkaline, and the corresponding absorption spectrum is also shown in the Figure.

Oxidation with oxygen

Kinetic experiments with oxygen-containing fluorescein solutions demonstrated that the permanent product was not formed directly upon photoexcitation but that intermediary stages were involved. The triplet form appears only during the flash and to a very low extent. Extinction measurements at the fluorescein peak in the visible revealed a more important decrease in fluorescein concentration during the flash than was accounted for by the triplet formed. After the flash the extinction reappeared without, however, reaching the original value. The sudden decrease in extinction was paralleled by a corresponding increase at about 430 mμ which faded out at the same rate as the rate of reappearance of the ground-state peak of fluorescein. This is illustrated in Fig. 43 which shows the extinction changes recorded at 428 and 491 mμ, upon flashing a 0.5 μM alkaline, air-saturated solution of fluorescein. The kinetic investigation indicates that oxygen is an efficient quencher of triplet molecules. There is apparently a certain, although low, tendency for a chemical reaction between oxygen and the triplet resulting in formation of an intermediary product exhibiting strong absorption around 430 mμ. The absorption spectrum of this intermediate, in alkaline solution, is shown in Fig. 44. This spectrum was obtained by extrapolation to complete conversion, under the assumption that the absorption of the intermediate is negligible at 491 mμ.

The intermediary product is identified, by its absorption spectrum, as the semi-oxidized form of fluorescein (the X form) also appearing in deaerated solutions as a result of the electron transfer processes (8) and (9). Hence, the mechanism for the oxygen attack probably should be expressed by the formula

$$T + O_2 \rightarrow S^{\cdot +} + O_2^{\cdot -}. \qquad (24)$$

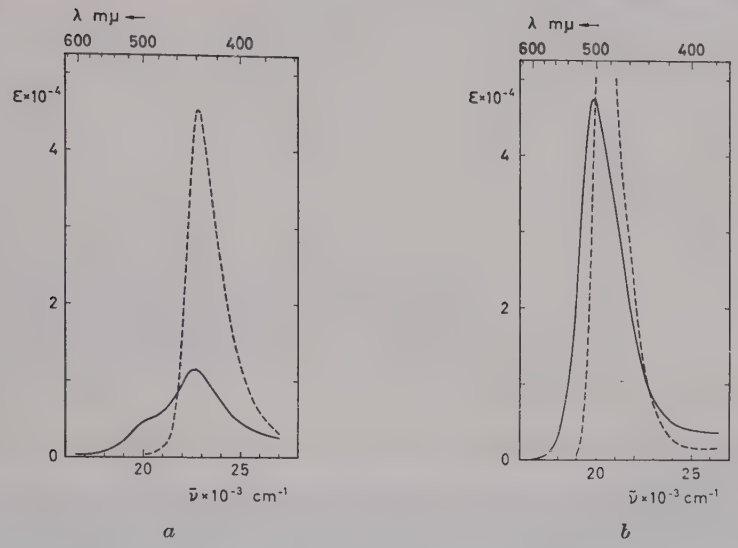


Fig. 42. Absorption spectrum of the pure oxidation product of fluorescein, obtained from a 10 μM fluorescein solution, 10^{-3} M with respect to ferric ions, exposed to 10 flashes of 6000 J ——. The spectrum of fluorescein is shown for comparison – – –.
(*a*) Acid solution, 0.025 M $HClO_4$. (*b*) The preceding solution after adding NaOH and removing the ferric hydroxide. NaOH concentration 0.01 M.

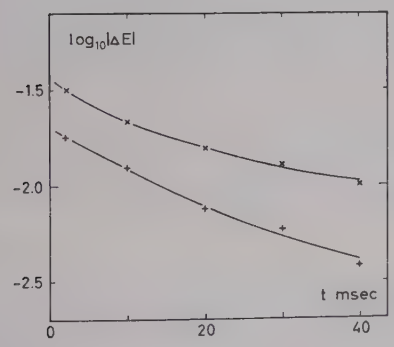


Fig. 43.

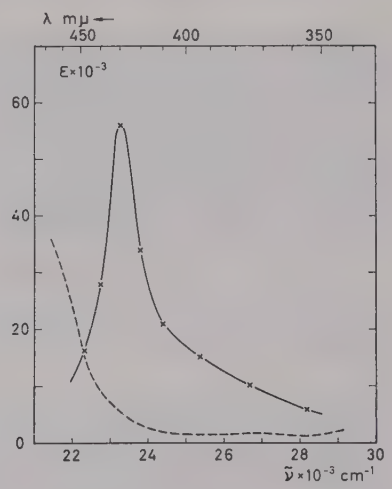


Fig. 44.

Fig. 43. Decay of the semi-oxidized form of fluorescein in oxygen-containing solutions. The logarithms of the extinction changes at 428 mμ +, and 491 mμ × are plotted against the time after flashing. Fluorescein concentration 0.50 μM in 0.01 M NaOH, air-saturated solution. The extinction change is negative at 491 mμ. Cuvette length 20 cm.

Fig. 44. The absorption spectrum of the semi-oxidized form of fluorescein as observed during flashing an air-saturated 1.0 μM solution of fluorescein in 0.01 M NaOH ×. The spectrum was obtained by extrapolation, setting the extinction coefficient of the semi-oxidized form equal to zero at 491 mμ. The absorption spectrum of fluorescein is shown for comparison – – –.

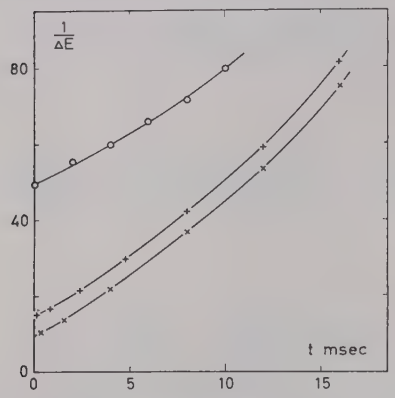


Fig. 45. Concentration dependence of the disappearance of the semi-oxidized form of fluorescein in air-saturated, alkaline solution (0.01 *M* NaOH). The reciprocal of the extinction change at 428 mμ is plotted against the time after flashing. Dye concentration 0.51 μM ○, 2.04 μM + and 4.05 μM ×. Cuvette length 20 cm.

The reduced oxygen probably rapidly forms stable products, and the semi-oxidized form is left behind.

The kinetics of the disappearance of the peak at 428 mμ were studied at different fluorescein concentrations. Fig. 45 shows results from measurements of 0.5 to 4 μM solutions of fluorescein in 0.01 *M* NaOH. The reciprocal of the extinction change is plotted against time. It is apparent that a second order reaction is involved, although the slopes increase somewhat with time. The only second order process that seems reasonable is the reaction

$$X + X \rightarrow \text{products} \qquad (k_{25}). \tag{25}$$

Taking the initial slope from Fig. 45 and the extinction coefficient at 428 mμ from Fig. 44, one obtains $k_{25} \approx 3 \times 10^9\ M^{-1}\ \text{sec}^{-1}$.

The kinetics of the appearance of the permanent products, measured at 530 mμ, showed that the coloured product was not formed at the rate determined by reaction (25). Equilibrium was attained only after a period of seconds. It has been assumed in another connection, p. 113, that X is apt to dimerize, and reaction (25) should in that case be rearranged to

$$X + X \rightarrow X_2 \rightarrow \text{products}.$$

The slow formation of permanent products would be explained if X_2 decomposes slowly. The elucidation of these ultimate steps requires further study.

Oxidation with ferric ions

The photo-oxidation of fluorescein in acid medium in the presence of ferric salts was studied. The decomposition is considerably higher than when oxygen is used as oxidant. The ferric ions react easily with triplet molecules. Fig. 46 shows extinction changes on flashing 0.5 μM fluorescein solutions in the presence and absence of ferric ions. The decay of the triplet molecules (observed at 540 mμ) is enhanced in the presence of ferric ions and the rate constant for the quenching reaction

$$T + \text{Fe}^{3+} \begin{matrix} \nearrow S + \text{Fe}^{3+} \\ \searrow X + \text{Fe}^{2+} \end{matrix} \qquad (k_{26}) \tag{26}$$

is found to be $1 \times 10^9\ M^{-1}\ \text{sec}^{-1}$.

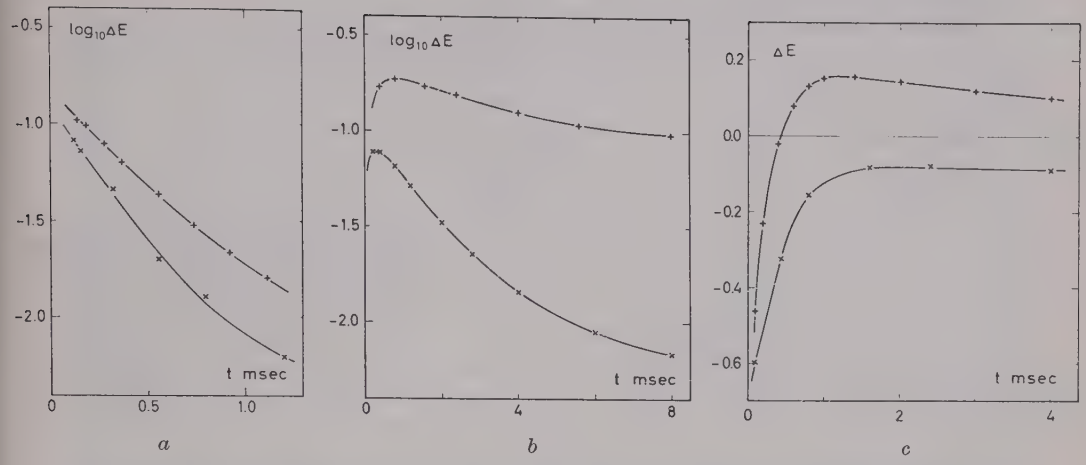


Fig. 46. Photo-oxidation of fluorescein in the presence of ferric ions, in acid solution (0.025 M $HClO_4$). Concentration of fluorescein 0.50 μM, of ferric ions 1.0 μM, cuvette length 60 cm. (*a*) The logarithm of the extinction change at 540 mμ, corresponding to the triplet absorption, plotted against the time after flashing ×. For comparison, the decay of the triplet in the absence of oxidant is also shown +. (*b*) The logarithm of the extinction change at 355 mμ, corresponding to the absorption of semi-reduced fluorescein, plotted against the time after flashing ×. For comparison, the change in the absence of oxidant is shown +. (*c*) The extinction change at 428 mμ, corresponding to the absorption of semi-oxidized fluorescein, plotted against the time after flashing, +. For comparison, the change in the absence of oxidant is shown ×.

The high irreversible decomposition, however, cannot be accounted for considering only reactions between triplet and ferric ions and has to be explained by attack on the semi-oxidized or semi-reduced forms of fluorescein formed at encounters between triplet molecules or between triplet and ground-state molecules. The semi-reduced form probably is reconverted to fluorescein by reaction with ferric ions. When only semi-oxidized molecules are left, these are expected to react further by reactions analogous to those encountered in the case of photo-oxidation with oxygen as oxidant. It is apparent from Fig. 46*b* that the semi-reduced form, responsible for the peak at 355 mμ, disappears very rapidly in the presence of ferric ions. Probably the reaction

$$R + Fe^{3+} \rightarrow S + Fe^{2+} \qquad (27)$$

occurs.

Fig. 46*c* shows the extinction changes at the absorption peak of the semi-oxidized form of fluorescein. The extinction increase in the presence of ferric ions indicates that quenching of triplet molecules by ferric ions at least in part involves a chemical reaction,

$$T + Fe^{3+} \rightarrow X + Fe^{2+}. \qquad (28)$$

Oxidation with peroxydisulphate ions

For the study of the photo-oxidation of fluorescein in alkaline solution, ferric salts could not be used and were replaced by potassium peroxydisulphate. This compound slowly oxidizes fluorescein even in the dark. No coloured decomposition products are observed in the dark reaction which apparently proceeds via a different reaction

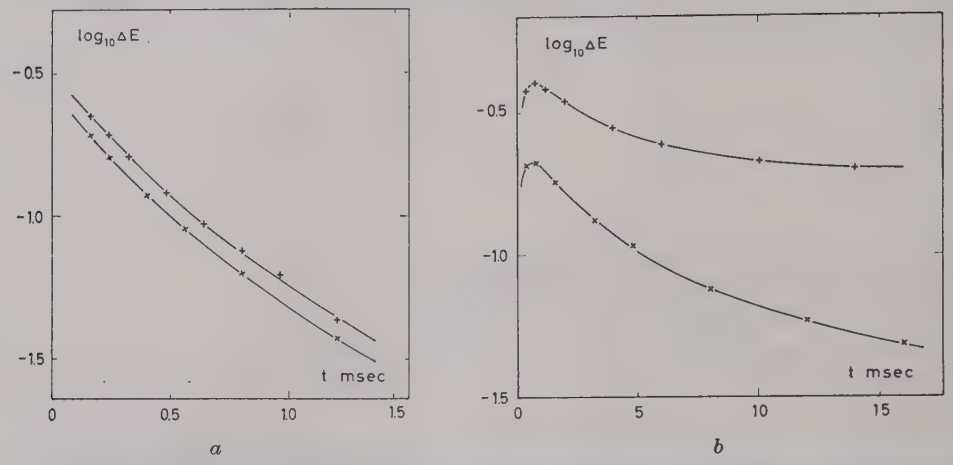


Fig. 47. Photo-oxidation of fluorescein in the presence of peroxydisulphate ions, in alkaline solution (0.01 *M* NaOH). Fluorescein concentration 2.0 μM, concentration of $K_2S_2O_8$ 100 μM, cuvette length 20 cm. The logarithms of the extinction changes are plotted against the time after flashing ×. For comparison, corresponding curves obtained in the absence of oxidant are also shown +. (*a*) Transient extinction at 600 mμ, corresponding to the triplet absorption. Dark oxidation during preparation of the solution accounts for the lower extinction change observed in the presence of oxidant. (*b*) Extinction change at 394 mμ, corresponding to the absorption of semi-reduced fluorescein.

mechanism than the light-induced oxidation. During the time of preparation of the solutions a decomposition of about 10 % occurred.

Potassium peroxydisulphate proved to be a very weak quencher of the triplet state. A flash photolysis experiment with a 2 μM solution of fluorescein in 0.01 *M* NaOH, at a $K_2S_2O_8$ concentration of 100 μM, was performed. Fig. 47 shows the transient extinction at 600 mμ, corresponding to the concentration of the triplet, compared to a similar run in the absence of oxidant. No effect of the peroxydisulphate ions is observed. Measurements of the extinction change at 394 mμ, the absorption peak of the semi-reduced radicals, show on the other hand that the oxidizing agent easily reacts with this species, which is formed at triplet-triplet or triplet-singlet encounters. The radical R probably is reconverted to fluorescein,

$$R + S_2O_8^{2-} \rightarrow S + SO_4^{2-} + \cdot SO_4^{-}. \qquad (29)$$

Chapter 5. Discussion

Lewis and co-workers related the phenomenon of phosphorescence to the existence of molecules in a triplet level, using fluorescein in boric acid 'glass' as a model system. This dye has since been studied by several other investigators, and the existence of a triplet state of the dye seems well established. The measurements of the magnetic susceptibility of the illuminated phosphor, carried out by Lewis and Calvin [15], were extended by Evans [16], who showed that the change in susceptibility was directly

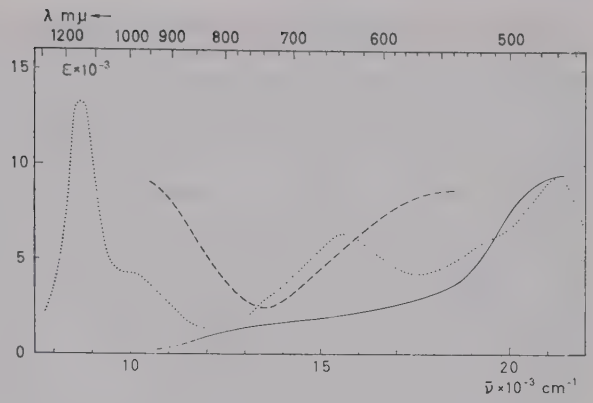


Fig. 48. Absorption spectra of the triplet state of fluorescein in aqueous solutions, at pH 1.7 —— and 12 ----, compared to the triplet spectrum in acid ether-ethanol glass, reported by Zanker and Miethke [20] ····.

related to the intensity of phosphorescence. The photomagnetism exhibited by fluorescein in glycerol at low temperatures was studied by Kortüm and Littmann [17]. The absorption spectrum of the phosphorescent state was measured by Lewis, Lipkin, and Magel [18], and later by Levshin and Laktionov [19]. Zanker and Miethke [20] reported the absorption spectrum of the triplet state of fluorescein in an ether-ethanol glass saturated with hydrochloric acid, at the temperature of liquid nitrogen, and Laffitte and Nouchi [21] carried out absorption measurements of the triplet in Plexiglass. The different studies on the triplet absorption, all performed in acid media, show comparatively good agreement.

In ordinary solutions direct evidence for the existence of a long-lived excited state of xanthene dyes is furnished by Boudin [22] and Kautsky [23], who observed the phosphorescence of such dyes in glycerol and water, respectively, at room temperature. Grossweiner and Zwicker [2] found diffuse absorption in the visible region on flashing eosin in oxygen-free water solution, and attributed this to a metastable state of eosin.

In the present investigation the existence of a long-lived, presumed triplet form of fluorescein in water solution has been established. The absorption spectra obtained in acid and alkaline solutions are compared in Fig. 48 to the spectrum in acid glassy medium reported by Zanker and Miethke. The strong infrared peak observed in the acid glass is absent in acid water solution, while a high absorption in the infrared appears in alkaline solution. The assignment of the spectrum in water to a metastable state is supported by the kinetic data, and it seems difficult to find another interpretation. The marked discrepancy between the spectra of the acid triplet in water and in glassy medium possibly is due to the rigidity of the glass. Terenin and Karyakin [24] have shown that when acridine and, for instance, oxalic acid are sublimed together onto a cool surface, the ionization equilibrium is disturbed on illumination with ultraviolet light and the fluorescence of the acridine base appears. The acridinium ion becomes a weaker acid in the excited singlet state and should not dissociate on illumination. The effect is explained by Terenin and Karyakin as due to excess vibrational energy overcoming the energy barrier between the two equilibrium positions of the proton in the rigid matrix. In the case of fluorescein in acid glass the dissociation should be favoured by the high acidity of the excited singlet state of fluorescein [11], and the appearance of the anionic form of the triplet would be explained.

Levshin and Laktionov found absorption bands at 415 and 350 mμ, not appearing in Zanker and Miethke's spectrum. The triplet spectrum in water solution does not reveal any bands at these wavelengths. In the present work the existence of a semi-oxidized and a semi-reduced form of fluorescein, formed at encounters between triplet molecules or between triplet and ground-state molecules was established. These radicals exhibit absorption maxima at 428 mμ and 355 mμ respectively in acid solution and might possibly account for the observed bands.

The study of the pH dependence of the absorption spectrum of the triplet in water solution showed that only two different spectra appear in the pH range 0 to 13. The triplet would be expected to exist as cation, quinonoid neutral molecule, mono-anion, and di-anion in this pH range (see p. 89). The absorption spectrum found in strongly alkaline solution can be attributed only to the di-anionic triplet. When the pH is decreased, the absorption spectrum is changed, at about pH 7, due to the appearance of the mono-anion. The mono-anion and the neutral molecule would be expected to have similar absorption spectra because the dissociation does not concern the light-absorbing groups, and the dissociation constant of the neutral molecule would for the same reason not be expected to change compared to that of unexcited fluorescein. Fig. 11 indicates a slight decrease in absorption when the pH is changed from 5.5 to 3.3. Fluorescein at pH 3.3 is present mainly in the uncoloured, lactonic form, and only to a small extent in the quinonoid neutral, the cationic, and the mono-anionic forms. The lactonic form is expected to be a very weak acid and consequently only the rates of exchange between this form and the quinonoid neutral form determine the equilibrium concentration of lactonic fluorescein in acid solution. The degree of conversion to the triplet state during flashing will depend upon these rates of exchange. The absorption decrease mentioned above indicates that the equilibrium between the two neutral forms is not established instantaneously. The rate constant for the rearrangement from the lactonic to the quinonoid form is estimated to be not less than 10^5 sec^{-1} and the rate constant for the reverse process correspondingly higher. In strongly acid solution the occurrence of the cationic form of the triplet would be expected. The absorption spectrum at pH 1.7 is, however, not different from that of the triplet mono-anion except for a slight absorption decrease. No new characteristic spectrum was apparent even at pH 0.

The dissociation equilibrium between the triplet mono-anion and di-anion was studied and it was shown that the dissociation constant is approximately the same as in the ground state. A similar behavior for acridine and other organic acid-base systems has been reported by Porter and Jackson [25]. They attributed the excitation to the triplet level to a π–π transition which does not influence the electron density at the point of proton attachment, while the excited singlet state which differs strongly in acid character from the ground state should be due to a n–π transition.

Kinetic measurements of the di-anionic form of the triplet showed this form to be unexpectedly long-lived, with a lifetime of 20 msec. In acid solution the lifetime is reduced to about 2 msec. Typical triplet lifetimes in ordinary liquids are of the order of 0.1 to 1 msec, the highest value being reported by Livingston and Tanner [26] who found a decay time of 10 msec for triplet anthracene in glycerol. Kautsky [23] found for fluorescein dyes in water solution a phosphorescence duration of about 10 msec, which fits well with the present results and also indicates that a considerable fraction of the metastable molecules are deactivated by re-emission of light.

The long life of the triplet may be associated with the use of water as solvent. Values reported mostly refer to organic solvents, and it is probable that the micro-

scopically rigid structure of water in addition to solvation effects would increase the lifetime of the triplet state.

The mono-anion and the neutral triplet molecule were expected to have the same lifetime, and this was confirmed by the kinetic measurements. At high acidities a change in lifetime due to the appearance of the triplet cation was expected but could not be detected.

The triplet lifetime is reduced when the fluorescein concentration is increased, due to quenching at encounters between triplet molecules or between triplet and ground-state molecules, Forms. (4.2) to (4.5). The concentration quenching is less pronounced in alkaline than in acid solution. This difference may be explained by the increased coulombic repulsion when going from the mono-anion to the di-anion. The carboxyl group is not involved in the quenching reaction and the negative charge of this group in the mono-anionic triplet therefore should not affect the kinetics to any appreciable extent. The results showed, in agreement with this, that the second order quenching constant was not influenced by pH in acid solution. The appearance of the triplet cation should cause a decrease in the deactivation efficiency due to the positive charge. Yet, even at the highest acidity studied, pH 0, no change in quenching constant was found. Since the dissociation constant of the triplet mono-anion is nearly equal to that of the corresponding ion in the ground state, the same would be expected to apply to the cationic forms. It is, on the other hand, unlikely that the triplet cation would both give the same decay kinetics and have the same absorption spectrum as the neutral triplet. The experimental results consequently furnish strong evidence for a shift of the pK_a value to below zero on excitation of the fluorescein cation to the triplet state.

The high values of the rate constants for the bimolecular quenching reactions, of the order of $10^9\ M^{-1}\ \text{sec}^{-1}$, are indicative of very low activation energies. These were not calculated because the influence of the relative orientation of the molecules on the reactions could not be easily estimated.

Oster and Adelman [27, 28] studied the photoreduction of fluorescein and derivatives and inferred that the reduction proceeds via a metastable state with a lifetime of about 0.1 msec. They also obtained the Stern-Volmer constant for the concentration quenching of the metastable state, corresponding to the ratio $(k_3+k_5)/k_1$, and for fluorescein gave a value of $15.1\times10^3\ M^{-1}$. The measurements were carried out at pH 7.0. The corresponding values resulting from the present study are 2 msec and $4\times10^6\ M^{-1}$ (calculated for the acid form of the triplet). The discrepancy may be due, at least in part, to the appearance of reactive radicals of fluorescein, formed according to mechanisms (4.8) and (4.9), not considered by Oster and Adelman.

The inclusion of electron transfer reactions, giving semi-reduced and semi-oxidized fluorescein radicals, is based upon the results of the kinetic measurements. A reaction analogous to mechanism (4.9), an electron dismutation between a molecule in an excited singlet level and an unexcited molecule, has been proposed by Weiss and Weil-Malherbe [29] as an explanation of the concentration quenching of fluorescence. According to them the reaction products should recombine after the quenching reaction, re-establishing the original conditions. The present work shows that reactions between the same kind of reaction products also may occur, complicating the picture. When triplet molecules are concerned, the electron transfer reaction will also occur in very dilute solutions because of the long life of the triplet. The reaction should be of importance from a photochemical standpoint, not only because two new reactive intermediates are introduced but also because the absorbed light energy

after the electron transfer reaction can no longer be wasted by phosphorescence or radiationless deactivation and is removed from chemical action only by the occurrence of back reactions.

The kinetic data show that the probability of formation of radicals upon encounter between a triplet and a singlet molecule is of the same order of magnitude as the probability of quenching without chemical reaction. In alkaline solution the rate constants are considerably lower than in acid solution, but still allow competition with the first order deactivation of the triplet at concentrations down to the μM range.

The assumption that the intermediary products are fluorescein radicals (or radical ions) is borne out by the photoreduction and photo-oxidation experiments. Other possibilities were also considered, for example formation of dimers of fluorescein in the ground state or in the triplet state (analogous to the excited dimers of pyrene [30]), but were not in accord with the experimental results.

The existence of semi-reduced forms of xanthene dyes is well established. In a study of the stationary state obtained on photoreduction of uranine with ferrous ions, Weiss [31] formulated the reaction as a one-electron reduction of the dye, followed by disproportionation of the semi-reduced radicals. Direct proof of the existence of the semi-reduced form of fluorescein was furnished by Imamura [6], who irradiated a deaerated, alkaline water-ethanol solution of fluorescein with visible light and found a strong absorption band in the violet (max. extinction coefficient 6.5×10^4 at 390–395 mμ), which he attributed to semi-reduced fluorescein. On admittance of oxygen the radical disappeared rapidly. In acid solution no band was observed. The present flash photolysis experiments give a maximum extinction coefficient for the semi-reduced form of 5×10^4, at 394 mμ, in good agreement with Imamura's values. The stability of the radical in alkaline solution was explained by Imamura as due to formation of a carbinol base. The present work shows that there is no pronounced change in the reaction kinetics of the semi-reduced form when going from alkaline to acid medium, and the hypothesis of carbinol formation is not supported.

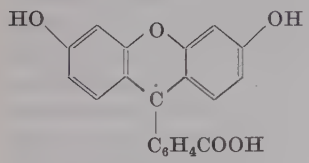
The photoreduction of eosin was studied by Tsepalov and Shliapintokh [32] by potentiometric and spectral methods, and the existence of a relatively stable intermediary product of the dye was revealed. This product disproportionates slowly— the reaction requires several seconds for completion— and the leuco-dye is formed. The slow disappearance of the radicals is confirmed by the present investigation. In a recent paper [33] the EPR spectrum of the intermediary product was determined and the results definitely showed that a radical species is formed, with the free electron situated principally on the central carbon atom of the molecule, a structure earlier proposed by Schenck [34].

The photoreduction of eosin with phenol in water solution has been studied by Grossweiner and Zwicker [2], using the flash photolysis method. Transient absorption bands were found at 382 and 398 mμ, corresponding to the peaks of the phenol-radical cation [35]. The semi-reduced eosin radicals which should be present in the irradiated solution were not revealed by the absorption measurements. On the other hand, Kato and Koizumi [3] showed that on flashing deaerated ethanol solutions three absorption bands appear, at 373, 410, and 450 mμ. They assigned the bands at 373 and 450 mμ to the semi-reduced form of eosin and the remaining band, which disappeared more rapidly than the others, to a molecular complex between the excited dye and ethanol. Their short communication does not permit a detailed comparison (the wavelength region for excitation was not stated), but there is a close similarity

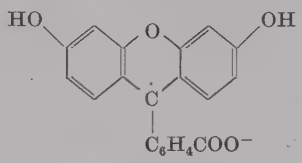
in kinetic behavior to that of fluorescein in water solution, as observed in the present investigation. The band at 410 mμ accordingly might be due to the semi-oxidized radical of eosin.

Polarographic measurements on fluorescein are reported by Delahay [36], who measured the half-wave potentials for cathodic reduction of fluorescein at different acidities. Below pH 7, one wave was observed, corresponding to a two-electron reduction, while in the pH range 7 to 10.5 two waves appeared, each corresponding to a one-electron reduction. The development of two waves was attributed to the formation of a semi-quinonoid form of fluorescein in alkaline solution. The present investigation shows that the semi-reduced fluorescein radical has a pK_a value of 9.5, and the development of two waves is probably explained by the appearance of the base form of the radical in alkaline solution.

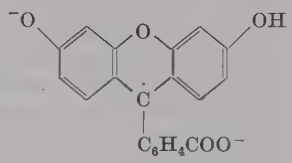
In the pH range 0 to 13 only one acid-base system of the semi-reduced radical is found. It is probable that the dissociation of the carboxyl proton will affect neither the absorption spectrum nor the reactivity of the semi-reduced form, and the corresponding pK_a value is not expected to be appreciably changed compared to that of unreacted fluorescein. It is believed, considering possible effects of charge, solvation, and mesomerism, that the neutral radical is a weaker acid than the fluorescein cation (which has a pK_a value of 2.2), and the following forms of the semi-reduced radicals consequently should appear in the pH range studied.



Neutral radical.



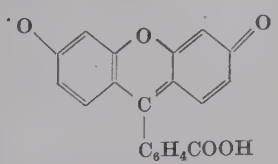
Radical mono-anion.



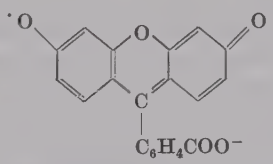
Radical di-anion.

The pK_a value of 9.5 is attributed to the dissociation of the radical mono-anion. The dissociation of the second phenolic hydrogen probably occurs at a pH above 13. This region is not amenable for study, however, because the oxygen bridge of fluorescein is ruptured in strongly alkaline solution.

While the existence of semi-reduced radicals of fluorescein is well substantiated in the literature, the same is not the case for the semi-oxidized radicals that also have to appear as a result of the electron transfer reaction. There does not seem to be any evidence reported for the existence of this form. The present work has revealed the existence of an intermediary product with an absorption band with maximum at 428 mμ, extinction coefficient 6×10^4. The band is not affected by pH in the pH region investigated, 0 to 13. The product is assumed to be the semi-oxidized fluorescein radical.



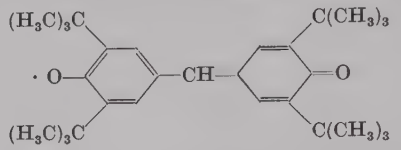
Neutral radical.



Radical anion.

This radical, by analogy with for example quinones, would be expected to add a

proton only in very strongly acid solution. The dissociation of the carboxyl proton should not influence the light-absorbing xanthene group, and the insensitivity of the product to changes in pH supports the assuption that it is the semi-oxidized radical. A similar phenoxy radical has been studied by Coppinger [37],



obtained by oxidation of 3,3′,5,5′-tetra-*tert*-butyl-4,4′-dihydroxydiphenylmethane. The radical is stable in *iso*-octane, even in the presence of oxygen. The absorption spectrum was measured, revealing a strong absorption band at 420 mμ (max. extinction coefficient 2×10^5). The presence of the bulky tertiary butyl substituents and the possibilities of mesomerism explain the exceptional stability of this radical. Aroxy radicals are generally known as strongly dehydrogenating agents, and the semi-oxidized fluorescein radicals would consequently be expected to be of some importance in photo-oxidation reactions.

The reactivity of the semi-oxidized radicals is reflected in the ease of dimerization, as revealed by the flash photolysis experiments, and expressed in Form. (4.11). It is believed that a bond is formed by the two free electrons and that a peroxy dimer is obtained. The possibilities of mesomerism would thereby be greatly reduced, explaining why no characteristic absorption of the dimer was found in the visible or near ultraviolet. The semi-oxidized radical might well occur to a small extent in equilibrium with the dimer, and the net reaction (4.13), which expresses the recovery of fluorescein by reaction between the semi-reduced and dimeric semi-oxidized forms of the dye, may in that case be divided into two reactions, a dissociation reaction of the dimer followed by an electron transfer between the semi-oxidized and semi-reduced radicals. The rate of recovery would be determined by the rate of dissociation, and the sluggishness of the reaction indicates that the dimer is dissociated to only a very low degree.

The rôle of the two radical forms of fluorescein in photochemical reactions is illustrated by the study of a few photoreduction and photo-oxidation reactions. A thorough discussion of the studied systems has to await a more detailed report of the reaction kinetics involved, and in this connection only the most important observations will be considered.

The study of the photoreduction with allyl-thiourea as reductant demonstrates the importance of the electron transfer reactions (4.8) and (4.9). Allyl-thiourea quenches the fluorescence of fluorescein (Stern-Volmer constant 15 M^{-1} at pH 7.0 [28]), possibly at the same time reducing the dye. This direct reduction cannot be appreciable at the low concentration of reductant used in the present investigation but probably accounts for the slightly lower triplet concentration observed during flashing a fluorescein solution when reductant is added (Fig. 38*a*). Allyl-thiourea also reacts with the triplet state, Form. (4.18), in part by a pure deactivation process and in part by a chemical reaction giving the semi-reduced form of fluorescein. It is probable that these reactions are about equally efficient, since the semi-reduced form appears to the same extent in the presence as in the absence of the reductant (Fig. 38*c*). Due to the low value of the rate constant k_{18}, the triplet-triplet transfer reaction (4.8) will also contribute to the triplet decay. The semi-oxidized fluorescein

radicals formed by this reaction react very rapidly with the reductant, as is apparent from Fig. 38 *b*. In an ordinary low-intensity photolysis the triplet-triplet electron transfer will not occur, and the corresponding triplet-singlet reaction, Form. (4.9), cannot compete with the direct reduction of the triplet unless the concentration of reductant is decreased considerably. In the photoreduction of fluorescein by agents of lower activity than allyl-thiourea this triplet-singlet reaction is expected to become increasingly important. The semi-oxidized radicals produced will probably react very readily with the reductant under formation of the original dye. The ultimate appearance of the leuco-dye then, as has been mentioned earlier, has to be attributed to a disproportionation of the semi-reduced radicals formed by reaction (4.9).

It has not been discussed whether the reduction of fluorescein proceeds via a hydrogen abstraction of allyl-thiourea or as an electron transfer process. Porter and Bridge [38] (see also Bridge and Maclean [39]), from a flash photolysis study of quinones as photosensitizers, concluded that when ethanol is used as reductant semi-quinones are formed by hydrogen abstraction. The true course of such reactions is debated [40], and it therefore would be of interest to determine the reaction path in the case of allyl-thiourea and fluorescein. If hydrogen abstraction occurs, the acid form of the semi-reduced fluorescein will be formed initially, and this form will dissociate in alkaline solution giving the conjugate base. The possibility of studying this reaction was eliminated in the present investigation by the addition of buffer systems, allowing establishment of equilibrium within the time of resolution of the kinetic measurements (this was done in order to simplify the treatment of the complex reaction kinetics), and further work is required to elucidate this reaction.

The first step in the irreversible photoreduction of fluorescein is the formation of the semi-reduced dye and the semi-oxidized reductant. If the semi-oxidized reductant is sufficiently stable, the reverse reaction may have time to occur before the product is consumed in other reactions. The inhibitory effect of *p*-phenylenediamine may be explained in this way. The semi-quinonoid form of *p*-phenylenediamine is the parent compound of the series of Wurster's salts, which are stable in moderately acid solution. The absorption spectra of these free radicals have been measured by Michaelis, Schubert, and Granick [41], who found an absorption region between 400 and 500 mμ for the semi-quinonoid form of *p*-phenylenediamine, max. extinction coefficient about 10^4. A strong absorption band in the ultraviolet (at 330 mμ) was observed by Lewis and Bigeleisen [42]. From the flash photolysis study of fluorescein in the presence of *p*-phenylenediamine the sum spectrum of the semi-reduced fluorescein and the semi-oxidized inhibitor was obtained, Fig. 39. The absorption bands of the semi-oxidized *p*-phenylenediamine, reported in the literature, are appearing in the transient spectrum. Due to the overlapping absorption of unchanged and semi-reduced fluorescein the shape of the radical bands cannot be determined in detail. Once the contribution of the sensitizer to the transient spectrum is known, it will be possible to obtain the absorption spectra of the unstable base forms of the Wurster's salts.

p-Phenylenediamine quenches the fluorescence of fluorescein (Stern-Volmer quenching constant 54 M^{-1} at pH 7.0 [28]). The quenching of fluorescence has been formulated by Weiss [43] as an electron transfer reaction between the quencher and the excited molecule. At the low concentrations used in the present investigation no fluorescence quenching will occur, and the inhibitor will instead attack the triplet molecules, by a mechanism analogous to that proposed by Weiss. The triplet quenching reaction apparently takes place with a vanishingly low activation energy, explaining the high efficiency of *p*-phenylenediamine as inhibitor. Since a true chem-

ical reaction is involved in the quenching process, the electron transfer reaction yielding ground-state fluorescein, Form. (4.21), has to occur with high probability at encounters between the reacted species; otherwise the semi-reduced fluorescein radicals would have the possibility of reacting with substrates present in the solution and the inhibitory effect would be lost. The flash photolysis experiments show that the reaction (4.21), as required, is mainly diffusion-controlled.

The effect of *p*-phenylenediamine on the photoreduction of fluorescein by allylthiourea has been studied by Adelman and Oster [28]. These authors concluded that the quenching reaction was diffusion controlled, and they determined the Stern-Volmer quenching constant for the triplet state. Their value, $1.06 \times 10^5\ M^{-1}$ at pH 7.0, is considerably lower than that obtained in the present work, $k_{20}/k_1 \approx 10^7\ M^{-1}$.

Reversible reductions involving chlorophyll and suitable reductants, for example ascorbic acid, analogous to the reduction described above, have been studied by Krasnovskiĭ [44]. In this case a photostationary state is directly observed even at low light intensities, due to the sluggishness of the chlorophyll-restoring reaction. If an electron acceptor, for example riboflavine, is present, the semi-reduced chlorophyll may react with the acceptor, the chlorophyll is restored, and the photosensitized reduction-oxidation process is completed.

That a photosensitizer may induce oxidation in this way is confirmed by the present study of the photo-oxidation of fluorescein in the presence of peroxydisulphate ions. The oxidation may in this case be considered as a photosensitized reaction, where the dye acts at the same time as the sensitizer and the electron donor. The results show (Fig. 47) that the triplet state of fluorescein does not react with the oxidant directly at the concentration studied, and the electron transfer reactions (4.8) and (4.9) will occur, giving semi-reduced and semi-oxidized fluorescein radicals. The semi-reduced radicals are rapidly reconverted to fluorescein by the peroxydisulphate, Form. (4.29), while the semi-oxidized radicals form irreversible oxidation products. This is the same scheme as that encountered in the photo-oxidation sensitized by chlorophyll, except that the triplet-triplet reaction (4.8) has to be considered, due to the high light intensities used.

At higher concentrations of peroxydisulphate the triplet state will probably be attacked directly by the oxidant, as was shown to be the case in the photo-oxidation of fluorescein by ferric ions in acid solution (Fig. 46). The triplet state is efficiently quenched by ferric ions, to a great extent by a chemical reaction giving ferrous ions and the semi-oxidized form of fluorescein. This reaction apparently is mainly diffusion controlled, and it is therefore probable that the direct oxidation of the fluorescein triplet constitutes the first step in photosensitized reactions involving ferric ions and fluorescein. Oster and Oster [45], in a study of the photoreduction of ferric salts by ethylenediaminetetraacetic acid with fluorescein as sensitizer, assumed that the first step is the reduction of the excited dye. This is not compatible with the present results.

The photo-oxidation of fluorescein by oxygen apparently proceeds in the same way as the oxidation by ferric ions. Oxygen has no quenching effect on the excited singlet state of the dye [46] but is an efficient quencher of the triplet state. The quenching by oxygen leads principally to the reappearance of ground-state fluorescein and differs in this respect from the quenching by ferric ions. This difference in behavior accounts for the very much higher irreversible photodecomposition observed in the presence of ferric ions. However, a chemical reaction between oxygen and the triplet also takes place, Form. (4.23), although only to a very low extent, and the semi-oxidized

form of fluorescein appears. An electron transfer reaction between oxygen and the excited sensitizer has been proposed by Weiss [47] as the first step in sensitized photo-oxidations, and is confirmed by the present investigation. The electron transfer mechanism was put forward as an alternative to Kautsky's hypothesis about the formation of oxygen in a singlet state [48]. Schenck [49] postulated the formation of an intermediate diradical peroxide complex by reaction of oxygen with the photosensitizer in the triplet state, in order to account for the kinetics observed in photosensitized diene oxidations. The existence of this complex was not revealed by the flash photolysis study and it consequently must have a lifetime lower than 10^{-5} sec.

The present results strongly indicate that the first step in autoxidations sensitized by xanthene dyes is the formation of the semi-oxidized dye and semi-reduced oxygen. The substrate to be oxidized will not have time left to react with the triplet molecules before these are quenched by the oxygen, unless it is a very strong reducing agent. The fluorescein radicals subsequently oxidize the substrate and the original dye is restored. The semi-reduced oxygen is a strong oxidizing agent and will probably also oxidize the substrate. Xanthene dyes are very efficient in producing the 'photodynamic effect' (for references, see [50]). This reaction is most often formulated as a reduction of the excited dye by the substrate, followed by a reaction between the reduced dye and oxygen. The present results indicate that the possibility of a direct attack of oxygen on the excited dye molecules also has to be considered.

SUMMARY

The purpose of this work has been to study the intermediary photoproducts of fluorescein and the reaction mechanisms underlying the photochemical activity of the dye.

The flash photolysis method provided the basis for the experimental investigation. A very intense flash light source was used for excitation. The absorption changes in the illuminated system were measured photoelectrically using a kinetic spectrophotometer, covering the spectral range 200–1200 mμ.

The intermediary products formed by flash photolysis of fluorescein in deaerated water solution were studied. Complete conversion to the triplet state was achieved. The dark reactions restored the initial conditions. The triplet decay was found to proceed in part by the quenching of the triplet state directly to the ground state. However, a chemical reaction between two triplet molecules or between one triplet and one unexcited fluorescein molecule also occurred, leading, by an electron transfer mechanism, to semi-reduced and semi-oxidized fluorescein radicals or radical ions. The electron transfer reaction was found to be reversible and the fluorescein was partially restored by the reverse reaction. The semi-oxidized form, however, also apparently dimerizes to a certain extent, and the dimers react but slowly with the semi-reduced form.

The reversible reaction was studied at different acidities, and it was shown that the reactions mentioned above governed the decay in the whole pH region investigated, pH 0–13.

The spectra of fluorescein in the triplet state, and in the semi-reduced and semi-oxidized states were measured at different acidities.

The photochemical importance of the electron transfer reaction discovered was illustrated by the study of a few photoreduction and photo-oxidation reactions.

The photoreduction of fluorescein with allyl-thiourea was studied. The reducing agent reacts with the triplet, as well as with the semi-oxidized fluorescein radicals formed by the electron transfer reaction between fluorescein molecules, mentioned above.

The photo-oxidation of fluorescein was studied using oxygen, ferric ions, and peroxydisulphate ions as oxidants.

It was shown that in the presence of oxygen the triplet is strongly quenched, and that semi-oxidized fluorescein radicals are formed in low yields. The radicals disappear by formation of irreversible oxidation products. The importance of this intermediary form as oxidizing agent in photosensitized autoxidations is stressed.

Ferric ions were shown to quench the triplet state efficiently, in part by an oxidation reaction. Ferric ions also attack the semi-reduced radicals formed by the electron transfer reaction between fluorescein molecules, mentioned above. The semi-oxidized radicals disappear yielding permanent photoproducts.

Potassium peroxydisulphate was found to be a poor quencher of the triplet state but reacts easily with the semi-reduced form of fluorescein, and permanent products are formed in high yields.

The inhibitory effect of *p*-phenylenediamine was also studied. Complete conversion of fluorescein to the semi-reduced state was achieved during flashing in the presence of inhibitor. The reduction was reversible and the recovery of fluorescein was very rapid, apparently proceeding by a diffusion controlled reaction. The semi-oxidized form of the inhibitor was also shown to appear.

It is inferred from the results that photochemical reactions involving xanthene dyes do not only proceed via the triplet state, but that radical forms of fluorescein, produced by reactions between dye molecules in the triplet and the ground state, in certain cases may play an even more important role.

The following reaction scheme summarizes the more important reactions originating from the triplet state of fluorescein, T. S denotes unchanged fluorescein, R and X the semi-reduced and semi-oxidized fluorescein radicals, respectively. A denotes a substrate, A_{red} and A_{ox} the semi-reduced and semi-oxidized forms of the substrate. The symbols are used irrespective of electrolytic dissociation.

Reactions in the absence of substrates,

$$T \rightarrow S \qquad (1)$$

$$T + T \rightarrow T + S \qquad (2)$$

$$T + S \rightarrow S + S \qquad (3)$$

$$T + T \rightarrow R + X \qquad (4), (8)$$

$$T + S \rightarrow R + X \qquad (5), (9)$$

$$R + X \rightarrow S + S \qquad (10)$$

$$X + X \rightarrow X_2 \qquad (11)$$

$$X_2 + 2R \rightarrow 4S. \qquad (13)$$

In the presence of substrates, the following chemical reactions will enter into competition with the above processes. The direct deactivation of the triplet to the ground state is not included in the scheme.

Reducing substrate,

$$T + A \rightarrow R + A_{ox} \qquad (18)$$

$$X + A \rightarrow S + A_{ox}. \qquad (19)$$

Oxidizing substrate,

$$T + A \rightarrow X + A_{red} \qquad (24), (28)$$

$$R + A \rightarrow S + A_{red}. \qquad (27), (29)$$

Substrate with inhibitory action,

$$T + A \rightarrow R + A_{ox} \tag{20}$$

$$R + A_{ox} \rightarrow S + A. \tag{21}$$

In a complementary investigation the dissociation stages of fluorescein in water solution were studied by determining the pH dependence of the absorption spectrum of the dye.

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